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(57) Abstract

A novel pathway for the synthesis of polyhydroxyalkanoates is provided. A method of synthesizing a recombinant polyhydroxyalkanoate monomer synthase is also provided. These recombinant polyhydroxyalkanoate synthases are derived from multifunctional fatty acid synthases or polyketide synthases and generate hydroxyacyl acids capable of polymerization by a polyhydroxyalkanoate synthase.

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# METABOLIC ENGINEERING OF POLY-HYDROXYALKANOATE MONOMER SYNTHASES

## Background of the Invention

Polyhydroxyalkanoates (PHAs) are one class of biodegradable polymers. The first identified member of the PHAs thermoplastics was polyhydroxybutyrate (PHB), the polymeric ester of D(-)-3-hydroxybutyrate.

The biosynthetic pathway of PHB in the gram negative bacterium *Alcaligenes eutrophus* is depicted in Figure 1. PHAs related to PHB differ in the structure of the pendant arm, R (Figure 2). For example, R=$CH_3$ in PHB, while R=$CH_2CH_3$ in polyhydroxyvalerate, and R=$(CH_2)_4CH_3$ in polyhydroxyoctanoate.

The genes responsible for PHB synthesis in *A. eutrophus* have been cloned and sequenced. (Peoples et al., J. Biol. Chem., 264, 15293 (1989); Peoples et al., J. Biol. Chem., 264, 15298 (1989)). Three enzymes: β-ketothiolase (*phb*A), acetoacetyl-CoA reductase (*phb*B), and PHB synthase (*phb*C) are involved in the conversion of acetyl-CoA to PHB. The PHB synthase gene encodes a protein of $M_r$=63,900 which is active when introduced into *E. coli* (Peoples et al., J. Biol. Chem., 264, 15298 (1989)).

Although PHB represents the archetypical form of a biodegradable thermoplastic, its physical properties preclude significant use of the homopolymer form. Pure PHB is highly crystalline and, thus, very brittle. However, unique physical properties resulting from the structural characteristics of the R groups in a PHA copolymer may result in a polymer with more desirable characteristics. These characteristics include altered crystallinity, UV weathering resistance, glass to rubber transition temperature ($T_g$), melting temperature of the crystalline phase, rigidity and durability (Holmes et al., EPO 00052 459; Anderson et al., Microbiol. Rev., 54, 450 (1990)). Thus, these polyesters behave as thermoplastics, with melting

temperatures of 50-180°C, which can be processed by conventional extension and molding equipment.

Traditional strategies for producing random PHA copolymers involve feeding short and long chain fatty acid monomers to bacterial cultures. However, this technology is limited by the monomer units which can be incorporated into a polymer by the endogenous PHA synthase and the expense of manufacturing PHAs by existing fermentation methods (Haywood et al., FEMS Microbiol. Lett., 57, 1 (1989); Poi et al., Int. J. Biol. Macromol., 12, 106 (1990); Steinbuchel et al., In: Novel Biomaterials from Biological Sources. D. Byron (ed.), MacMillan, NY (1991); Valentin et al., Appl. Microbiol. Biotechnical, 36, 507 (1992)).

The production of diverse hydroxyacylCoA monomers for homo- and co-polymeric PHAs also occurs in some bacteria through the reduction and condensation pathway of fatty acids. This pathway employs a fatty acid synthase (FAS) which condenses malonate and acetate. The resulting β-keto group undergoes three processing steps, β-keto reduction, dehydration, and enoyl reduction, to yield a fully saturated butyryl unit. However, this pathway provides only a limited array of PHA monomers which vary in alkyl chain length but not in the degree of alkyl group branching, saturation, or functionalization along the acyl chain.

The biosynthesis of polyketides, such as erythromycin, is mechanistically related to formation of long-chain fatty acids. However, polyketides, in contrast to FASs, retain ketone, hydroxyl, or olefinic functions and contain methyl or ethyl side groups interspersed along an acyl chain comparable in length to that of common fatty acids. This asymmetry in structure implies that the polyketide synthase (PKS), the enzyme system responsible for formation of these molecules, although mechanistically related to a

FAS, results in an end product that is structurally very different than that of a long chain fatty acid.

Because PHAs are biodegradable polymers that have the versatility to replace petrochemical-based thermoplastics, it is desirable that new, more economic methods be provided for the production of defined PHAs. Thus, what is needed are methods to produce recombinant PHA monomer synthases for the generation of PHA polymers.

## Summary of the Invention

The present invention provides a method of preparing a polyhydroxyalkanoate synthase. The method comprises introducing an expression cassette into a non-plant eukaryotic cell. The expression cassette comprises a DNA molecule encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in the non-plant eukaryotic cell. The DNA molecule encoding the polyhydroxyalkanoate synthase is then expressed in the cell. Thus, another embodiment of the invention provides a purified, isolated recombinant polyhydroxybutyrate synthase.

Another embodiment of the invention is a method of preparing a polyhydroxyalkanoate polymer. The method comprises introducing a first expression cassette and a second expression cassette into a eukaryotic cell. The first expression cassette comprises a DNA segment encoding a fatty acid synthase in which the dehydrase activity has been inactivated that is operably linked to a promoter functional in the eukaryotic cell. The second expression cassette comprises a DNA segment encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in the eukaryotic cell. The DNA segments in the expression cassettes are expressed in the cell so as to yield a polyhydroxyalkanoate polymer.

Another embodiment of the invention is a baculovirus expression cassette comprising a nucleic acid molecule encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in an insect cell.

The present invention also provides an expression cassette comprising a nucleic acid molecule encoding a polyhydroxyalkanoate monomer synthase operably linked to a promoter functional in a host cell. The nucleic acid molecule comprises a plurality of DNA segments. Thus, the nucleic acid molecule comprises at least a first and a second DNA segment. No more than one DNA segment is derived from the *ery*A gene cluster of *Saccharopolyspora erythraea*. The first DNA segment encodes a first module and the second DNA segment encodes a second module, wherein the DNA segments together encode a polyhydroxyalkanoate synthase.

Also provided is an isolated and purified DNA molecule. The DNA molecule comprises a plurality of DNA segments. Thus, the DNA molecule comprises at least a first and a second DNA segment. The first DNA segment encodes a first module and the second DNA segment encodes a second module. No more than one DNA segment is derived from the *ery*A gene cluster of *Saccharopolyspora erythraea*. Together the DNA segments encode a recombinant polyhydroxyalkanoate monomer synthase. A preferred embodiment of the invention employs a first DNA segment derived from the *vep* gene cluster of *Streptomyces*. Another preferred embodiment of the invention employs a second DNA segment derived from the *tyl* gene cluster of *Streptomyces*.

Yet another embodiment of the invention is a method of providing a polyhydroxyalkanoate monomer. The method comprises introducing a DNA molecule into a host cell. The DNA molecule comprises a DNA segment encoding a recombinant

polyhydroxyalkanoate monomer synthase operably linked to a promoter functional in the host cell. The DNA encoding the recombinant polyhydroxyalkanoate monomer synthase, which synthase comprises at least a first module and a second module, is expressed in the host cell so as to generate a polyhydroxyalkanoate monomer.

Also provided is a method of preparing a polyhydroxyalkanoate polymer. The method comprises introducing a first DNA molecule and a second DNA molecule into a host cell. The first DNA molecule comprises a DNA segment encoding a recombinant polyhydroxyalkanoate monomer synthase. The recombinant polyhydroxyalkanoate monomer synthase comprises a plurality of modules. Thus, the monomer synthase comprises at least a first module and a second module. The first DNA molecule is operably linked to a promoter functional in a host cell. The second DNA molecule comprises a DNA segment encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in the host cell. The DNAs encoding the recombinant polyhydroxyalkanoate monomer synthase and polyhydroxyalkanoate synthase are expressed in the host cell so as to generate a polyhydroxyalkanoate polymer.

Yet another embodiment of the invention is an isolated and purified DNA molecule. The DNA molecule comprises a plurality of DNA segments. That is, the DNA molecule comprises at least a first and a second DNA segment. The first DNA segment encodes a fatty acid synthase and the second DNA segment encodes a module of a polyketide synthase. A preferred embodiment of the invention employs a second DNA segment encoding a module which comprises a β-ketoacyl synthase amino-terminal to an acyltransferase

which is amino-terminal to a ketoreductase which is amino-terminal to an acyl carrier protein which is amino-terminal to a thioesterase.

The invention also provides a method of preparing a polyhydroxyalkanoate monomer. The method comprises introducing a DNA molecule comprising a plurality of DNA segments into a host cell. Thus, the DNA molecule comprises at least a first and a second DNA segment. The first DNA segment encodes a fatty acid synthase operably linked to a promoter functional in the host cell. The second DNA segment encodes a polyketide synthase. The second DNA segment is located 3' to the first DNA segment. The first DNA segment is linked to the second DNA segment so that the encoded protein is expressed as a fusion protein. The DNA molecule is then expressed in the host cell so as to generate a polyhydroxyalkanoate monomer.

Another embodiment of the invention is an expression cassette comprising a DNA molecule comprising a DNA segment encoding a fatty acid synthase and a polyhydroxyalkanoate synthase.

Also provided is a method of providing a polyhydroxyalkanoate monomer synthase. The method comprises introducing an expression cassette into a host cell. The expression cassette comprises a DNA molecule encoding a polyhydroxyalkanoate monomer synthase operably linked to a promoter functional in the host cell. The monomer synthase comprises a plurality of modules. Thus, the monomer synthase comprises at least a first and second module which together encode the monomer synthase.

A further embodiment of the invention is an isolated and purified DNA molecule comprising a DNA segment which encodes a *Streptomyces venezuelae* polyhydroxyalkanoate monomer synthase, a biologically active variant or subunit thereof. Preferably, the DNA segment encodes a polypeptide having an amino acid sequence comprising SEQ ID NO:2. Preferably, the DNA segment comprises

SEQ ID NO:1. The DNA molecules of the invention are double stranded or single stranded. A preferred embodiment of the invention is a DNA molecule that has at least about 70%, more preferably at least about 80%, and even more preferably at least about 90%, identity to the DNA segment comprising SEQ ID NO:1, e.g., a "variant" DNA molecule. A variant DNA molecule of the invention can be prepared by methods well known to the art, including oligonucleotide-mediated mutagenesis. See Adelman et al., DNA, 2, 183 (1983) and Sambrook et al., Molecular Cloning: A Laboratory Manual (1989).

The invention also provides an isolated, purified polyhydroxyalkanoate monomer synthase, e.g., a polypeptide having an amino acid sequence comprising SEQ ID NO:2, a biologically active subunit, or a biologically active variant thereof. Thus, the invention provides a variant polypeptide having at least about 80%, more preferably at least about 90%, and even more preferably at least about 95%, identity to the polypeptide having an amino acid sequence comprising SEQ ID NO:2. A preferred variant polypeptide, or subunit of a polypeptide, of the invention includes a variant or subunit polypeptide having at least about 10%, more preferably at least about 50% and even more preferably at least about 90%, the activity of the polypeptide having the amino acid sequence comprising SEQ ID NO:2. Preferably, a variant polypeptide of the invention has one or more conservative amino acid substitutions relative to the polypeptide having the amino acid sequence comprising SEQ ID NO:2. For example, conservative substitutions include aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids. The biological activity of a polypeptide of the invention can be measured by methods well known to the art.

As used herein, a "linker region" is an amino acid sequence present in a multifunctional protein which is less well conserved in amino acid sequence than an amino acid sequence with catalytic activity.

As used herein, an "extender unit" catalytic or enzymatic domain is an acyl transferase in a module that catalyzes chain elongation by adding 2-4 carbon units to an acyl chain and is located carboxy-terminal to another acyl transferase. For example, an extender unit with methylmalonylCoA specificity adds acyl groups to a methylmalonylCoA molecule.

As used herein, a "polyhydroxyalkanoate" or "PHA" polymer includes, but is not limited to, linked units of related, preferably heterologous, hydroxyalkanoates such as 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxycaproate, 3-hydroxyheptanoate, 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxyundecanoate, and 3-hydroxydodecanoate, and their 4-hydroxy and 5-hydroxy counterparts.

As used herein, a "Type I polyketide synthase" is a single polypeptide with a single set of iteratively used active sites. This is in contrast to a Type II polyketide synthase which employs active sites on a series of polypeptides.

As used herein, a "recombinant" nucleic acid or protein molecule is a molecule where the nucleic acid molecule which encodes the protein has been modified *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been modified.

As used herein, a "multifunctional protein" is one where two or more enzymatic activities are present on a single polypeptide.

As used herein, a "module" is one of a series of repeated units in a multifunctional protein, such as a Type I polyketide synthase or a fatty acid synthase.

As used herein, a "premature termination product" is a product which is produced by a recombinant multifunctional protein which is different than the product produced by the non-recombinant multifunctional protein. In general, the product produced by the recombinant multifunctional protein has fewer acyl groups.

As used herein, a DNA that is "derived from" a gene cluster, is a DNA that has been isolated and purified *in vitro* from genomic DNA, or synthetically prepared on the basis of the sequence of genomic DNA.

As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a DNA or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide, so that it can be sequenced, replicated and/or expressed. Moreover, the DNA may encode more than one recombinant Type I polyketide synthase and/or fatty acid synthase. For example, "an isolated DNA molecule encoding a polyhydroxyalkanoate monomer synthase " is RNA or DNA containing greater than 7, preferably 15, and more preferably 20 or more sequential nucleotide bases that encode a biologically active polypeptide, fragment, or variant thereof, that is complementary to the non-coding, or complementary to the coding strand, of a polyhydroxyalkanoate monomer synthase RNA, or hybridizes to the RNA or DNA encoding the polyhydroxyalkanoate monomer synthase and remains stably bound under stringent conditions, as defined by methods well known to the art, e.g., in Sambrook et al., *supra*.

## Brief Description of the Figures

Figure 1: The PHB biosynthetic pathway in *A. eutrophus*.

Figure 2: Molecular structure of common bacterial PHAs. Most of the known PHAs are polymers of 3-hydroxy acids possessing the general formula shown. For example, R=$CH_3$ in PHB, R=$CH_2CH_3$ in polyhydroxyvalerate (PHV), and R=$(CH_2)_4CH_3$ in polyhydroxyoctanoate (PHO).

Figure 3: Comparison of the natural and recombinant pathways for PHB synthesis. The three enzymatic steps of PHB synthesis in bacteria involving 3-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase are shown on the left. The two enzymatic steps involved in PHB synthesis in the pathway in *Sf*21 cells containing a rat fatty acid synthase with an inactivated dehydrase domain (ratFAS206) are shown on the right.

Figure 4: Schematic diagram of the molecular organization of the *tyl* polyketide synthase (PKS) gene cluster. Open arrows correspond to individual open reading frames (ORFs) and numbers above an ORF denote a multifunctional module or synthase unit (SU). AT=acyltransferase; ACP=acyl carrier protein; KS=β-ketoacyl synthase; KR=ketoreductase; DH=dehydrase; ER=enoyl reductase; TE=thioesterase; MM=methylmalonylCoA; M=malonyl CoA; EM=ethylmalonyl CoA. Module 7 in *tyl* is also known as Module F.

Figure 5: Schematic diagram of the molecular organization of the *met* PKS gene cluster.

Figure 6: Strategy for producing a recombinant PHA monomer synthase by domain replacement.

Figure 7: (A) 10% SDS-PAGE gel showing samples from various stages of the purification of PHA synthase; lane 1,

molecular weight markers; lane 2, total protein of uninfected insect cells; lane 3, total protein of insect cells expressing a rat FAS ( 200 kDa; Joshi et al., Biochem J., 296, 143 (1993)); lane 4, total protein of insect cells expressing PHA synthase; lane 5, soluble protein from sample in lane 4; lane 6, pooled hydroxylapatite (HA) fractions containing PHA synthase. (B) Western analysis of an identical gel using rabbit-α-PHA synthase antibody as probe. Bands designated with arrows are: a, intact PHB synthase with N-terminal alanine at residue 7 and serine at residue 10 (A7/S10); b, 44 kDa fragment of PHB synthase with N-terminal alanine at residue 181 and asparagine at residue 185 (A181/N185); c, PHB synthase fragment of approximately 30 kDa apparently blocked based on resistance to Edman degradation; d, 22 kDa fragment with N-terminal glycine at residue 187 (G187). Band d apparently does not react with rabbit-α-PHB synthase antibody (B, lane 6). The band of similar size in B, lane 4 was not further identified.

Figure 8: N-terminal analysis of PHA synthase purified from insect cells. (a) The expected N-terminal 25 amino acid sequence of *A. eutrophus* PHA synthase. (b&c) The two N-terminal sequences determined for the *A. eutrophus* PHA synthase produced in insect cells. The bolded sequences are the actual N-termini determined.

Figure 9: Spectrophotometric scans of substrate, 3-hydroxybutyrate CoA (HBCoA) and product, CoA. The wavelength at which the direct spectrophotometric assays were carried out (232 nm) is denoted by the arrow; substrate, HBCoA (•) and product, CoA (◦).

Figure 10: Velocity of the hydrolysis of HBCoA as a function of substrate concentration. Assays were carried out in 40 or 200 µl assay volumes with enzyme concentration remaining constant at 0.95 mg/ml (3.8 µg/40µl assay). Velocities were calculated from the linear portions of the assay curves subsequent to the characteristic lag

period. The substrate concentration at half-optimal velocity, the apparent $K_m$ value, was estimated to be 2.5 mM from this data.

Figure 11: Double reciprocal plot of velocity versus substrate concentration. The concave upward shape of this plot is similar to results obtained by Fukui et al. (Arch. Microbiol., 110, 149 (1976)) with granular PHA synthase from *Z. ramigera*.

Figure 12: Velocity of the hydrolysis of HBCoA as a function of enzyme concentration. Assays were carried out in 40 µl assay volumes with the concentration HBCoA remaining constant at 8 µM.

Figure 13: Specific activity of PHA synthase as a function of enzyme concentration.

Figure 14: pH activity curve for soluble PHA synthase produced using the baculovirus system. Reactions were carried out in the presence of 200 mM $P_i$. Buffers of pH<10 were prepared with potassium phosphate, while buffers of pH>10 were prepared with the appropriate proportion of $Na_3PO_4$.

Figure 15: Assays of the hydrolysis of HBCoA with varying amounts of PHA synthase. Assays were carried out in 40 µl assay volumes with the concentration of HBCoA remaining constant at 8 µM. Initial $A_{232}$ values, originally between 0.62 and 0.77, were normalized to 0.70. Enzyme amounts used in these assays were, from the upper-most curve, 0.38, 0.76, 1.14, 1.52, 1.90, 2.28, 2.66, 3.02, 3.42, 7.6, and 15.2 µg, respectively.

Figure 16: SDS/PAGE analysis of proteins synthesized at various time-points during infection of *Sf*21 cells. Approximately 0.5 mg of total cellular protein from various samples was fractionated on a 10% polyacrylamide gel. Samples include: uninfected cells, lanes 1-4, days 0, 1, 2, 3 respectively; infection with BacPAK6::phbC alone,

lanes 5-8, days 0, 1, 2, 3 respectively; infection with baculoviral clone containing ratFAS206 alone, lanes 9-12, days 0, 1, 2, 3 respectively; and ratFAS206 and BacPAK6 infected cells, lanes 13-16 days 0, 1, 2, 3, respectively. A=mobility of FAS, B=mobility of PHA synthase. Molecular weight standard lanes are marked M.

Figure 17: Gas chromatographic evidence for PHB accumulation in *Sf*21 cells. Gas chromatograms from various samples are superimposed. PHB standard (Sigma) is chromatogram #7 showing a propylhydroxybutyrate elution time of 10.043 minutes (s, arrow). The gas chromatograms of extracts of the uninfected (#1); singly infected with ratFAS206 (#2, day 3); and singly infected with PHA synthase (#3, day 3) are shown at the bottom of the figure. Gas chromatograms of extracts of dual-infected cells at day 1 (#4), 2 (#5), and 3 (#6) are also shown exhibiting a peak eluting at 10.096 minutes (x, arrow). The peak of dual-infected, day 3 extract (#6) was used for mass spectrometry (MS) analysis.

Figure 18: Gas-chromatography-mass spectrometry analysis of PHB. The characteristic fragmentation of propylhydroxybutyrate at m/z of 43, 60, 87, and 131 is shown. A) standard PHB from bacteria (Sigma), and B) peak X from ratFAS206 and BacPAK6: phbC baculovirus infected, day 3 (#6, Figure 17) *Sf*21 cells expressing rat FAS dehydrase inactivated protein and PHA synthase.

Figure 19: Map of the *vep* (*Streptomyces venezuelae* polyene encoding) gene cluster.

Figure 20: Plasmid map of pDHS502.

Figure 21: Plasmid map of pDHS505.

Figure 22: Cloning protocol for pDHS505.

Figure 23: Nucleotide sequence (SEQ ID NO:1) and corresponding amino acid sequence (SEQ ID NO:22) of the *vep* ORFI.

## Detailed Description of the Invention

The invention described herein can be used for the production of a diverse range of biodegradable PHA polymers through genetic redesign of DNA encoding a FAS or *Streptomyces spp.* Type I PKS polypeptide to provide a recombinant PHA monomer synthase. Different PHA synthases can then be tested for their ability to polymerize the monomers produced by the recombinant PHA synthase into a biodegradable polymer. The invention also provides a method by which various PHA synthases can be tested for their specificity with respect to different monomer substrates.

The potential uses and applications of PHAs produced by PHA monomer synthases and PHA synthases includes both medical and industrial applications. Medical applications of PHAs include surgical pins, sutures, staples, swabs, wound dressings, blood vessel replacements, bone replacements and plates, stimulation of bone growth by piezoelectric properties, and biodegradable carrier for long-term dosage of pharmaceuticals. Industrial applications of PHAs include disposable items such as baby diapers, packaging containers, bottles, wrappings, bags, and films, and biodegradable carriers for long-term dosage of herbicides, fungicides, insecticides, or fertilizers.

In animals, the biosynthesis of fatty acids *de novo* from malonyl-CoA is catalyzed by FAS. For example, the rat FAS is a homodimer with a subunit structure consisting of 2505 amino acid residues having a molecular weight of 272,340 Da. Each subunit consists of seven catalytic activities in separate physical domains (Amy et al., Proc. Natl. Acad. Sci. USA, 86, 3114 (1989)). The physical location of six of the catalytic activities, ketoacyl synthase (KS), malonyl/acetyltransferase (M/AT), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP), and thioesterase (TE), has been established by (1) the identification of the various active site residues within the overall amino acid sequence by isolation of

catalytically active fragments from limited proteolytic digests of the whole FAS, (2) the identification of regions within the FAS that exhibit sequence similarity with various monofunctional proteins, (3) expression of DNA encoding an amino acid sequence with catalytic activity to produce recombinant proteins, and (4) the identification of DNA that does not encode catalytic activity, i.e., DNA encoding a linker region. (Smith et al., Proc. Natl. Acad. Sci. USA, 73, 1184 (1976); Tsukamoto et al., J. Biol. Chem., 263, 16225 (1988); Rangan et al., J. Biol. Chem., 266, 19180 (1991)).

The seventh catalytic activity, dehydrase (DH), was identified as physically residing between AT and ER by an amino acid comparison of FAS with the amino acid sequences encoded by the three open reading frames of the *ery*A polyketide synthase (PKS) gene cluster of *Saccharopolyspora erythraea*. The three polypeptides that comprise this PKS are constructed from "modules" which resemble animal FAS, both in terms of their amino acid sequence and in the ordering of the constituent domains (Donadio et al., Gene, 111, 51 (1992); Benh et al., Eur. J. Biochem., 204, 39 (1992)).

One embodiment of the invention employs a FAS in which the DH is inactivated (FAS DH-). The FAS DH- employed in this embodiment of the invention is preferably a eukaryotic FAS DH- and, more preferably, a mammalian FAS DH-. The most preferred embodiment of the invention is a FAS where the active site in the DH has been inactivated by mutation. For example, Joshi et al. (J. Biol. Chem., 268, 22508 (1993)) changed the $His^{878}$ residue in the rat FAS to an alanine residue by site directed mutagenesis. *In vitro* studies showed that a FAS with this change (ratFAS206) produced 3-hydroxybutyrylCoA as a premature termination product from acetyl-CoA, malonyl-CoA and NADPH.

As shown below, a FAS DH- effectively replaces the β-ketothiolase and acetoacetyl-CoA reductase activities of the natural pathway by producing D(-)-3-hydroxybutyrate as a premature termination product, rather than the usual 16-carbon product, palmitic acid. This premature termination product can then be incorporated into PHB by a PHB synthase (See Example 2).

Another embodiment of the invention employs a recombinant *Streptomyces spp.* PKS to produce a variety of β-hydroxyCoA esters that can serve as monomers for a PHA synthase. One example of a DNA encoding a Type I PKS is the *ery*A gene cluster, which governs the synthesis of erythromycin aglycone deoxyerythronolide B (DEB). The gene cluster encodes six repeated units, termed modules or synthase units (SUs). Each module or SU, which comprises a series of putative FAS-like activities, is responsible for one of the six elongation cycles required for DEB formation. Thus, the processive synthesis of asymmetric acyl chains found in complex polyketides is accomplished through the use of a programmed protein template, where the nature of the chemical reactions occurring at each point is determined by the specificities in each SU.

Two other Type I PKS are encoded by the *tyl* (tylosin) (Figure 4) and *met* (methymycin) (Figure 5) gene clusters. The macrolide multifunctional synthases encoded by *tyl* and *met* provide a greater degree of metabolic diversity than that found in the *ery*A gene cluster. The PKSs encoded by the *ery*A gene cluster only catalyze chain elongation with methylmalonylCoA, as opposed to *tyl* and *met* PKSs, which catalyze chain elongation with malonylCoA, methylmalonylCoA and ethylmalonylCoA. Specifically, the *tyl* PKS includes two malonylCoA extender units and one ethylmalonylCoA extender unit, and the *met* PKS includes one malonylCoA extender unit. Thus, a preferred embodiment of the invention includes, but is

not limited to, replacing catalytic activities encoded in *met* PKS open reading frame 1 (ORF1) to provide a DNA encoding a protein that possesses the required keto group processing capacity and short chain acylCoA ester starter and extender unit specificity necessary to provide a saturated β-hydroxyhexanoylCoA or unsaturated β-hydroxyhexenoylCoA monomer.

In order to manipulate the catalytic specificities within each module, DNA encoding a catalytic activity must remain undisturbed. To identify the amino acid sequences between the amino acid sequences with catalytic activity, the "linker regions," amino acid sequences of related modules, preferably those encoded by more than one gene cluster, are compared. Linker regions are amino acid sequences which are less well conserved than amino acid sequences with catalytic activity. Witkowski et al., Eur. J. Biochem., 198, 571 (1991).

In an alternative embodiment of the invention, to provide a DNA encoding a Type I PKS module with a TE and lacking a functional DH, a DNA encoding a module F, containing KS, MT, KR, ACP, and TE catalytic activities, is introduced at the 3' end of a DNA encoding a first module (Figure 6). Module F introduces the final (R)-3-hydroxyl acyl group at the final step of PHA monomer synthesis, as a result of the presence of a TE domain. DNA encoding a module F is not present in the *ery*A PKS gene cluster (Donadio et al., *supra*, 1991).

A DNA encoding a recombinant monomer synthase is inserted into an expression vector. The expression vector employed varies depending on the host cell to be transformed with the expression vector. That is, vectors are employed with transcription, translation and/or post-translational signals, such as targeting signals, necessary for efficient expression of the genes in various host cells

into which the vectors are introduced. Such vectors are constructed and transformed into host cells by methods well known in the art. See Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor (1989). Preferred host cells for the vectors of the invention include insect, bacterial, and plant cells. Preferred insect cells include *Spodoptera frugiperda* cells such as *Sf*21, and *Trichoplusia ni* cells. Preferred bacterial cells include *Escherichia coli*, *Streptomyces* and *Pseudomonas*. Preferred plant cells include monocot and dicot cells, such as maize, rice, wheat, tobacco, legumes, carrot, squash, canola, soybean, potato, and the like.

Moreover, the appropriate subcellular compartment in which to locate the enzyme in eukaryotic cells must be considered when constructing eukaryotic expression vectors. Two factors are important: the site of production of the acetyl-CoA substrate, and the available space for storage of the PHA polymer. To direct the enzyme to a particular subcellular location, targeting sequences may be added to the sequences encoding the recombinant molecules.

The baculovirus system is particularly amenable to the introduction of DNA encoding a recombinant FAS or a PKS monomer synthase because an increasing variety of transfer plasmids are becoming available which can accommodate a large insert, and the virus can be propagated to high titers. Moreover, insect cells are adapted readily to suspension culture, facilitating relatively large scale recombinant protein production. Further, recombinant proteins tend to be produced exclusively as soluble proteins in insect cells, thus, obviating the need for refolding, a task that might be particularly daunting in the case of a large multifunctional protein. The *Sf*21/baculovirus system has routinely expressed milligram quantities of catalytically active recombinant fatty acid synthase. Finally, the baculovirus/insect cell system provides the ability to construct and

analyze different synthase proteins for the ability to polymerize monomers into unique biodegradable polymers.

A further embodiment of the invention is the introduction of at least one DNA encoding a PHA synthase and a DNA encoding a PHA monomer synthase into a host cell. Such synthases include, but are not limited to, *A. eutrophus* 3-hydroxy, 4-hydroxy, and 5-hydroxy alkanoate synthases, *Rhodococcus ruber* $C_3$-$C_5$ hydroxyalkanoate synthases, *Pseudomonas oleororans* $C_6$-$C_{14}$ hydroxyalkanoate synthases, *P. putida* $C_6$-$C_{14}$ hydroxyalkanoate synthases, *P. aeruginosa* $C_5$-$C_{10}$ hydroxyalkanoate synthases, *P. resinovorans* $C_4$-$C_{10}$ hydroxyalkanoate synthases, *Rhodospirillum rubrum* $C_4$-$C_7$ hydroxyalkanoate synthases, *R. gelatinorus* $C_4$-$C_7$, *Thiocapsa pfennigii* $C_4$-$C_8$ hydroxyalkanoate synthases, and *Bacillus megaterium* $C_4$-$C_5$ hydroxyalkanoate synthases.

The introduction of DNA(s) encoding more than one PHA synthase may be necessary to produce a particular PHA polymer due to the specificities exhibited by different PHA synthases. As multifunctional proteins are altered to produce unusual monomeric structures, synthase specificity may be problematic for particular substrates. Although the *A. eutrophus* PHB synthase utilizes only C4 and C5 compounds as substrates, it appears to be a good prototype synthase for initial studies since it is known to be capable of producing copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate (Kunioka et al., Macromolecules, 22, 694 (1989)) as well as copolymers of 3-hydroxyvalerate, 3-hydroxybutyrate, and 5-hydroxyvalerate (Doi et al., Macromolecules, 19, 2860 (1986)). Other synthases, especially those of *Pseudomonas aeruginosa* (Timm et al., Eur. J. Biochem., 209, 15 (1992)) and *Rhodococcus ruber* (Pieper et al., FEMS Microbiol. Lett., 96, 73 (1992)), can also be employed in the practice of the invention. Synthase specificity may be alterable through molecular biological methods.

In yet another embodiment of the invention, a DNA encoding a FAS and a PHA synthase can be introduced into a single expression vector, obviating the need to introduce the genes into a host cell individually.

A further embodiment of the invention is the generation of a DNA encoding a recombinant multifunctional protein, which comprises a FAS, of either eukaryotic or prokaryotic origin, and a PKS module F. Module F will carry out the final chain extension to include two additional carbons and the reduction of the β-keto group, which results in a (R)-3-hydroxy acyl CoA moiety.

To produce this recombinant protein, DNA encoding the FAS TE is replaced with a DNA encoding a linker region which is normally found in the ACP-KS interdomain region of bimodular ORFs. DNA encoding a module F is then inserted 3′ to the DNA encoding the linker region. Different linker regions, such as those described below, which vary in length and amino acid composition, can be tested to determine which linker most efficiently mediates or allows the required transfer of the nascent saturated fatty acid intermediate to module F for the final chain elongation and keto reduction steps. The resulting DNA encoding the protein can then be tested for expression of long chain β-hydroxy fatty acids in insect cells, such as S*f*21 cells, or *Streptomyces*, or *Pseudomonas*. The expected 3-hydroxy C-18 fatty acid can serve as a potential substrate for PHA synthases which are able to accept long chain alkyl groups. A preferred embodiment of the invention is a FAS that has a chain length specificity between 4-22 carbons.

Examples of linker regions that can be employed in this embodiment of the invention include, but are not limited to, the ACP-KS linker regions encoded by the *tyl* ORFI ($ACP_1$-$KS_2$; $ACP_2$-

$KS_3$), and ORF3 ($ACP_5$-$KS_6$), and *eryA* ORFI ($ACP_1$-$KS_1$; $ACP_2$-$KS_2$), ORF2 ($ACP_3$-$KS_4$) and ORF3 ($ACP_5$-$KS_6$).

This approach can also be used to produce shorter chain fatty acid groups by limiting the ability of the FAS unit to generate long chain fatty acids. Mutagenesis of DNA encoding various FAS catalytic activities, starting with the KS, may result in the synthesis of short chain (R)-3-hydroxy fatty acids.

The PHA polymers are then recovered from the biomass. Large scale solvent extraction can be used, but is expensive. An alternative method involving heat shock with subsequent enzymatic and detergent digestive processes is also available (Byron, Trends Biotechnical, 5, 246 (1987); Holmes, In: Developments in Crystalline Polymers, D.C. Bassett (ed), pp. 1-65 (1988)). PHB and other PHAs are readily extracted from microorganisms by chlorinated hydrocarbons. Refluxing with chloroform has been extensively used; the resulting solution is filtered to remove debris and concentrated, and the polymer is precipitated with methanol or ethanol, leaving low-molecular-weight lipids in solution. Longer-side-chain PHAs show a less restricted solubility than PHB and are, for example, soluble in acetone. Other strategies adopted include the use of ethylene carbonate and propylene carbonate as disclosed by Lafferty et al. (Chem. Rundschau, 30, 14 (1977)) to extract PHB from biomass. Scandola et al., (Int. J. Biol. Microbiol., 10, 373 (1988)) reported that 1 M HCl-chloroform extraction of *Rhizobium meliloti* yielded PHB of $M_w$ = $6 \times 10^4$ compared with $1.4 \times 10^6$ when acetone was used.

Methods are well known in the art for the determination of the PHB or PHA content of microorganisms, the composition of PHAs, and the distribution of the monomer units in the polymer. Gas chromatography and high-pressure liquid chromatography are widely used for quantitative PHB analysis. See Anderson et al. Microbiol. Rev., 54, 450 (1990) for a review of such methods. NMR

techniques can also be used to determine polymer composition, and the distribution of monomer units.

The invention has been described with reference to various specific and preferred embodiments and will be further described by reference to the following detailed examples. It is understood however, that there are many extensive variations, and modifications on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope of the present invention.

## I. Experimental Procedures

### Materials and Methods

**Materials.** Sodium R-(-)-3-hydroxybutyrate, coenzyme-A, ethylchloroformate, pyridine and diethyl ether were purchased from Sigma Chemical Co. Amberlite IR-120 was purchased from Mallinckrodt Inc. 6-O-(N-Heptylcarbamoyl)methyl α-D-glucopyranoside (Hecameg) was obtained from Vegatec (Villeejuif, France). Two-piece spectrophotometer cells with pathlengths of 0.1 (#20/0-Q-1) and 0.01 cm (#20/0-Q-0.1) were obtained from Starna Cells Inc., (Atascadero, CA). Rabbit anti-*A. eutrophus* PHA synthase antibody was a gracious gift from Dr. F. Srienc and S. Stoup (Biological Process Technology Institute, University of Minnesota). *Sf*21 cells and *T. ni* cells were kindly provided by Greg Franzen (R&D Systems, Minneapolis, MN) and Stephen Harsch (Department of Veterinary Pathobiology, University of Minnesota), respectively.

Plasmid pFAS206 and a recombinant baculoviral clone encoding FAS206 (Joshi et al., J. Biol. Chem., 268, 22508 (1993)) were generous gifts of A. Joshi and S. Smith. Plasmid pAet41 (Peoples et al.,

J. Biol. Chem., 264, 15298, (1989)), the source of the *A. eutrophus* PHB synthase, was obtained from A. Sinskey. Baculovirus transfer vector, pBacPAK9, and linearized baculoviral DNA, were obtained from Clontech Inc. (Palo Alto, CA). Restriction enzymes, T4 DNA ligase, *E. coli* DH5α competent cells, molecular weight standards, lipofectin reagent, Grace's insect cell medium, fetal bovine serum (FBS), and antibiotic/antimycotic reagent were obtained from GIBCO-BRL (Grand Island, NY). Tissue culture dishes were obtained from Corning Inc. Spinner flasks were obtained from Bellco Glass Inc. Seaplaque agarose GTG was obtained from FMC Bioproducts Inc.

**Methods.**

**Preparation of R-3HBCoA.** R-(-)-3 HBCoA was prepared by the mixed anhydride method described by Haywood et al., FEMS Microbiol. Lett., 57, 1 (1989). 60 mg (0.58 mmol) of R-(-)-3 hydroxybutyric acid was freeze dried and added to a solution of 72 mg of pyridine in 10 ml diethyl ether at 0°C. Ethylchloroformate (100 mg) was added, and the mixture was allowed to stand at 4°C for 60 minutes. Insoluble pyridine hydrochloride was removed by centrifugation. The resulting anhydride was added, dropwise with mixing, to a solution of 100 mg coenzyme-A (0.13 mmol) in 4 ml 0.2 M potassium bicarbonate, pH 8.0 at 0°C. The reaction was monitored by the nitroprusside test of Stadtman, Meth. Enzymol., 3, 931 (1957), to ensure sufficient anhydride was added to esterify all the coenzyme-A. The concentration of R-3-HBCoA was determined by measuring the absorbance at 260 nm ($\epsilon$ = 16.8 $mM^{-1}$ $cm^{-1}$; 18).

**Construction of pBP-phbC.** The *phbC* gene (approximately 1.8 kb) was excised from pAet41 (Peoples et al., J. Biol. Chem., 264, 15293 (1989)) by digestion with *Bst*BI and *Stu*I, purified as described by Williams et al. (Gene, 109, 445 (1991)), and ligated to pBacPAK9

digested with *Bst*BI and *Stu*I. This resulted in pBP-phbC, the baculovirus transfer vector used in formation of recombinant baculovirus particles carrying *phbC*.

**Large scale expression of PHA synthase.** A 1 L culture of *T. ni* cells ($1.2x10^6$ cells/ml) in logarithmic growth was infected by the addition of 50 ml recombinant viral stock solution ( $2.5x10^8$ pfu/ml) resulting in a multiplicity of infection (MOI) of 10. This infected culture was split between two Bellco spinners (350 ml/500 ml spinner, 700 ml/1 L spinner) to facilitate oxygenation of the culture. These cultures were incubated at 28°C and stirred at 60 rpm for 60 hours. Infected cells were harvested by centrifugation at 1000 x g for 10 minutes at 4°C. Cells were flash-frozen in liquid $N_2$ and stored in 4 equal aliquots, at -80°C until purification.

**Insect cell maintenance and recombinant baculovirus formation.** *Sf*21 cells were maintained at 26-28°C in Grace's insect cell medium supplemented with 10% FBS, 1.0% pluronic F68, and 1.0% antibiotic/antimycotic (GIBCO-BRL). Cells were typically maintained in suspension at 0.2 - 2.0 x $10^6$/ml in 60 ml total culture volume in 100 ml spinner flasks at 55-65 rpm. Cell viability during the culture period was typically 95-100%. The procedures for use of the transfer vector and baculovirus were essentially those described by the manufacturer (Clontech, Inc.). Purified pBP-phbC and linearized baculovirus DNA were used for cotransfection of *Sf*21 cells using the liposome mediated method (Felgner et al., Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)) utilizing Lipofectin (GIBCO-BRL). Four days later cotransfection supernatants were utilized for plaque purification. Recombinant viral clones were purified from plaque assay plates containing 1.5% Seaplaque GTG after 5-7 days at 28°C. Recombinant viral clone stocks were then amplified in T25-flask cultures (4 ml, $3x10^6$/ml on day 0) for 4 days; infected cells were determined by their morphology and size and then screened by SDS/PAGE using 10%

polyacrylamide gels (Laemmli, Nature, 227, 680 (1970)) for production of PHA synthase.

**Purification of PHA synthase from BTI-TN-5B1-4 *T. ni* cells.** Purification of PHA synthase was performed according to the method of Gerngross et al., Biochemistry, 33, 9311 (1994) with the following alterations. One aliquot ( 110 mg protein) of frozen cells was thawed on ice and resuspended in 10 mM KPi (pH 7.2), 5% glycerol, and 0.05% Hecameg (Buffer A) containing the following protease inhibitors at the indicated final concentrations: benzamidine (2mM), phenylmethylsulfonyl fluoride (PMSF, 0.4 mM), pepstatin (2 mg/ml), leupeptin (2.5 mg/ml), and Na-*p*-tosyl-l-lysine chloromethyl ketone (TLCK, 2 mM). EDTA was omitted at this stage due to its incompatibility with hydroxylapatite (HA). This mixture was homogenized with three series of 10 strokes each in two Thomas homogenizers while partially submerged in an ice bath and then sonicated for 2 minutes in a Branson Sonifier 250 at 30% cycle, 30% power while on ice. All subsequent procedures were carried out at 4°C.

The lysate was immediately centrifuged at 100000 x g in a Beckman 50.2Ti rotor for 80 minutes, and the resulting supernatant (10.5 ml, 47 mg) was immediately filtered through a 0.45 mm Uniflow filter (Schleicher and Schuell Inc., Keene, N.H.) to remove any remaining insoluble matter. Aliquots of the soluble fraction (1.5 ml, 7 mg) were loaded onto a 5 ml BioRad Econo-Pac HTP column that had been equilibrated with Buffer A (+ protease inhibitor mix) attached to a BioRad Econo-system, and the column was washed with 30 ml Buffer A. All chromatographic steps were carried out at a flow rate of 0.8 ml/minute. PHA synthase was eluted from the HA column with a 32 x 32 ml linear gradient from 10 to 300 mM KPi.

Fraction collection tubes were prepared by addition of 30 ml of 100 mM EDTA to provide a metalloprotease inhibitor at 1 mM

immediately after HA chromatography. PHA synthase was eluted in a broad peak between 110-180 mM KPi. Fractions (3 ml) containing significant PHA synthase activity were pooled and stored at 0°C until the entire soluble fraction had been run through the chromatographic process. Pooled fractions then were concentrated at 4°C by use of a Centriprep-30 concentrator (Amicon) to 3.8 mg/ml. Aliquots (0.5 ml) were either flash-frozen and stored in liquid $N_2$ or glycerol was added to a final concentration of 50% and samples (1.9 mg/ml) were stored at -20°C.

**Western analysis.** Samples of *T. ni* cells were fractionated by SDS-PAGE on 10% polyacrylamide gels, and the proteins then were transferred to 0.2 mm nitrocellulose membranes using a BioRad Transblot SD Semi-Dry electrophoretic transfer cell according to the manufacturer. Proteins were transferred for 1 hour at 15 V. The membrane was rinsed with doubly distilled $H_2O$, dried, and treated with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween) and 3% nonfat dry milk to block non-specific binding sites. Primary antibody (rabbit anti-PHA synthase) was applied in fresh blocking solution and incubated at 25°C for 2 hours. Membranes were then washed four times for 10 minutes with PBS-Tween followed by the addition of horseradish peroxidase-conjugated goat-anti-rabbit antibody (Boehringer-Mannheim) diluted 10,000X in fresh blocking solution and incubated at 25°C for 1 hour. Membranes were washed finally in three changes (10 minutes) of PBS, and the immobilized peroxidase label was detected using the chemiluminescent LumiGLO substrate kit (Kirkegaard and Perry, Galthersburg, MD) and X-ray film.

**N-terminal analysis.** Approximately 10 mg of purified PHA synthase was run on a 10% SDS-polyacrylamide gel, transferred to PVDF (Immobilon-PSQ, Millipore Corporation, Bedford, MA), stained with Amido Black, and sequenced on a 494 Procise Protein

Sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, California).

**Double-infection protocol.** Four 100 ml spinner flasks were each inoculated with 8 x $10^7$ cells in 50 ml of fresh insect medium. To flask 1, an additional 20 ml of fresh insect medium was added (uninfected control); to flask 2, 10 ml BacPAK6::*phbC* viral stock ( 1 x $10^8$ pfu/ml) and 10 ml fresh insect medium were added; to flask 3, 10 ml BacPAK6::FAS206 viral stock ( 1 x $10^8$ pfu/ml) and 10 ml fresh insect medium were added; and to flask 4, 10 ml BacPAK6::*phbC* viral stock ( 1 x $10^8$ pfu/ml) and 10 ml BacPAK6::FAS206 viral stock ( 1 x $10^8$ pfu/ml) were added. These viral infections were carried out at a multiplicity of infection of approximately 10. Cultures were maintained under normal growth conditions and 15 ml samples were removed at 24, 48, and 72 hour time points. Cells were collected by gentle centrifugation at 1000 x g for 5 minutes, the medium was discarded, and the cells were immediately stored at –70°C.

**PHA synthase assays.** Coenzyme A released by PHA synthase in the process of polymerization was monitored precisely as described by Gerngross et al. (*supra*) using 5,5'-dithiobis (2-nitrobenzoic acid, DTNB) (Ellman, Arch. Biochem. Biophys., 82, 70 (1959)).

The presence of HBCoA was monitored spectrophotometrically. Assays were performed at 25°C in a Hewlett Packard 8452A diode array spectrophotometer equipped with a water jacketed cell holder. Two-piece Starna Spectrosil spectrophotometer cells with pathlengths of 0.1 and 0.01 cm were employed to avoid errors arising from the compression of the absorbance scale at higher values. Absorbance was monitored at 232 nm, and $E_{232}$nm of 4.5 X 103 $M^{-1}$ $cm^{-1}$ was used in calculations. One unit (U) of enzyme is the amount required to hydrolyze 1 mmol of substrate $minute^{-1}$. Buffer

(0.15 M KPi, pH 7.2) and substrate were equilibrated to 25°C and then combined in an Eppendorf tube also at 25°C. Enzyme was added and mixed once in the pipet tip used to transfer the entire mixture to the spectrophotometer cell. The two piece cell was immediately assembled, placed in the spectrophotometer with the cell holder (type CH) adapted for the standard 10 mm path length cell holder of the spectrophotometer. Manipulations of sample, from mixing to initiation of monitoring, took only 10-15 seconds. Absorbance was continually monitored for up to 10 minutes. Calibration of reactions was against a solution of buffer and enzyme (no substrate) which lead to absorbance values that represented substrate only.

**PHB assay.** PHB was assayed from *Sf*21 cell samples according to the propanolysis method of Riis et al., J. Chromo., 445, 285 (1988). Cell pellets were thawed on ice, resuspended in 1 ml cold $ddH_2O$ and transferred to 5 ml screwtop test tubes with teflon seals. 2 ml $ddH_2O$ was added, the cells were washed and centrifuged and then 3 ml of acetone were added and the cells washed and centrifuged. The samples were then dessicated by placing them in a 94°C oven for 12 hours. The following day 0.5 ml of 1,2-dichloroethane, 0.5 ml acidified propanol (20 ml HCl, 80 ml 1-propanol) and 50 ml benzoic acid standard were added and the sealed tubes were heated to 100°C in a boiling water bath for 2 hours with periodic vortexing. The tubes were cooled to room temperature and the organic phase was used for gas-chromatographic (GC) analysis using a Hewlett Packard 5890A gas-chromatograph equipped with a Hewlett Packard 7673A automatic injector and a fused silica capillary column, DB-WAX 30W of 30 meter length. Positive samples were further subjected to GC-mass spectrometric (MS) analysis for the presence of propylhydroxybutyrate using a Kratos MS25 GC/MS. The following parameters were used: source temperature, 210°C; voltage, 70eV; and accelerating voltage, 4 KeV.

**Catalytic activities.**

Ketoacyl synthase (KS) activity was assessed radiochemically by the condensation-$^{14}CO_2$ exchange reaction (Smith et al., PNAS USA, 73, 1184 (1976)).

Transferase (AT) activity was assayed, using malonyl-CoA as donor and pantetheine as acceptor, by determining spectrophotometrically the free CoA released in a coupled ATP citrate-lyase-malate dehydrogenase reaction (see, Rangen et al., J. Biol. Chem., 266, 19180 (1991).

Ketoreductase (KR) was assayed spectrophotometrically at 340 nm: assay systems contained 0.1 M potassium phosphate buffer (pH 7), 0.15 mM NADPH, enzyme and either 10 mM *trans*-1-decalone or 0.1 mM acetoacetyl-CoA substrate.

Dehydrase (DH) activity was assayed spectrophotometrically at 270 nm using S-DL-β-hydroxybutyryl N-acetylcysteamine as substrate (Kumar et al., J. Biol. Chem., 245, 4732 (1970)).

Enoyl reductase (ER) activity was assayed spectrophotometrically at 340 nm essentially as described by Stromeyer (J. Biol. Chem., 254, 8159 (1979)); the assay system contained 0.1 M potassium phosphate buffer (pH 7), 0.15 mM NADPH, 0.375 mM crotonoyl-CoA, 20 μM CoA and enzyme.

Thioesterase (TE) activity was assessed radiochemically by extracting and assaying the [$^{14}$C]palmitic acid formed from [1-$^{14}$C]palmitoyl-CoA during a 3 minute incubation Smith, Meth. Enzymol., 71C, 181 (1981); the assay was in a final volume of 0.1 ml, 25 mM potassium phosphate buffer (pH 8), 20 μM [1-$^{14}$C]palmitoyl-CoA (20 nCi) and enzyme.

Assay of overall fatty acid synthase activity was performed spectrophotometrically as described previously by Smith et al. (Meth.

Enzymol., 35, 65 (1975)). All enzyme activities were assayed at 37°C except the transferase, which was assayed at 20°C. Activity units indicate nmol of substrate consumed/minute. All assays were conducted, at a minimum, at two different protein concentrations with the appropriate enzyme and substrate blanks included.

**Example 1**

Expression of *A. eutrophus* PHA synthase using a baculovirus system.

Recent work has shown that PHA synthase from *A. eutrophus* can be overexpressed in *E. coli*, in the absence of 3-ketothiolase and acetoacetyl-CoA reductase (Gerngross et al. *supra*) and can be expressed in plants (See Poirier et al., Biotech, 13, 142 (1995) for a review). Isolation of the soluble form of PHA synthase provides opportunities to examine the mechanistic details of the priming and initiation reactions. Because the baculovirus system has been successful for the expression of a number of prokaryotic genes as soluble proteins, and insect cells, unlike bacterial expression systems, carry out a wide array of posttranslational modifications, the baculovirus expression system appeared ideal for the expression of large quantities of soluble PHA synthase, a protein that must be modified by phosphopantetheine in order to be catalytically active (Gerngross et al., *supra*).

**Purification of PHA synthase**. The purification procedure employed for PHA synthase is a modification of Gerngross et al. (*supra*) involving the elimination of the second liquid chromatographic step and inclusion of a protease-inhibitor cocktail in all buffers. All steps were carried out on ice or at 4°C except where noted. Frozen cells were thawed on ice in 10 ml of Buffer A (10 mM KPi, pH 7.2, 0.5% glycerol, and 0.05% Hecameg) and then immediately homogenized prior to centrifugation and HA chromatography.

The results of these efforts are summarized in Table 1 and Figure 7. A prominent band at 64 kDa is visible in total, soluble, and HA eluate protein samples fractionated by SDS/PAGE (lanes 4, 5, and 6 of Figure 7, respectively). The initial specific activity of the isolated PHA synthase was 20-fold higher than previous attempts at expression and purification of this polypeptide. Approximately 1000 units of PHB synthase have been purified, based on calculations from the direct spectrophotometric assay detailed below, with an overall recovery of activity of 70%. The large proportion of synthase present in the membrane fraction, and the fact that over 90% of the initial activity was found in the soluble fraction, suggests either that the synthase in the membrane fraction is in an inactive form or that the direct assay is not applicable to the initial, 12 U/mg, crude extract.

Table 1: Purification of PHA Synthase

| sample | total units | vol (mL) | protein (mg) | protein (mg/ml) | specific activity | recovery |
|---|---|---|---|---|---|---|
| total protein | 1430 | 11.5 | 113 | 9.8 | 12.7 | 100 |
| soluble protein | 1340 | 10.5 | 47 | 4.5 | 28.6 | 93 |
| pooled HA fractions | 1020 | 7.9 | 30 | 3.8 | 34.2 | 71 |

N-terminal sequencing of the 64 kDa protein confirmed its identity as PHA synthase (Figure 8). Two prominent N-termini, at amino acid residue 7 (alanine) and residue 10 (serine) were obtained in a 3:2 ratio. This heterogeneous N-terminus presumably is the result of aminopeptidase activity. Western analysis using a rabbit-

anti-PHA synthase antibody corroborated the results of the sequencing and indicated the presence of at least three bands that resulted from proteolysis of PHA synthase (Figure 7B, Lanes 4-6). The antibody was specific for PHA synthase since neither *T. ni* nor baculoviral proteins showed reactivity (Figure 7B, Lanes 2 and 3). N-terminal protein sequencing (Figure 8) showed directly that the 44 kDa (band b) and 32 kDa (band d) proteins were derived from PHA synthase (fragments beginning at A181/N185 and at G387, respectively). The 35-40 kDa (band c) protein gave low sequencing yields and may contain a blocked N-terminus. Inspection of Figure 7B suggests that most degradation occurs following cell disruption since the total protein sample for this gel (lane 4) was prepared by boiling intact cells directly in SDS sample buffer while the HA sample (lane 6) went through the purification procedure described above.

**Assay of Synthase Activity.** Due to the significant level of expression obtained using the baculovirus system, the synthase activity could be assayed spectrophotometrically by monitoring hydrolysis of the thioester bond at 232 nm, the wavelength at which there is a maximum decrease in absorbance upon hydrolysis. The difference between substrate (HBCoA) and product (CoA) at this wavelength is shown in Figure 9. Absorbance of HBCoA and CoA at 232 nm occurs at a trough between two well separated peaks. Assays were carried out at pH 7.2 for comparative analysis with previous studies (Gerngross et al., *supra*). Substrate (R-(-)3-HBCoA) substrate for these studies was prepared using the mixed anhydride method (Haywood et al., *supra*), and its concentration was determined by measuring $A_{260}$. The short pathlength cells (0.1 cm and 0.01 cm) allowed use of relatively high reaction concentrations while conserving substrate and enzyme. Assay results showed an initial lag period of 60 seconds prior to the linear decrease in $A_{232}$, and velocities were determined from the slope of these linear regions of

the assay curves. The length of the lag period was variable and was inversely related to enzyme concentration. These data are consistent with those using PHA synthase purified from *E. coli* (Gerngross et al., *supra*).

Figures 10 and 11 show the V versus S and 1/V versus 1/S plots, respectively. The double reciprocal plot was concave upward which is similar to results obtained from studies of the granular PHA synthase from *Zooglea ramigera* (Fukui et al., Arch. Microbiol., 110, 149 (1976)) and suggests a complex reaction mechanism. Examinations of velocity and specific activity as a function of enzyme concentration are shown in Figures 12 and 13. These results confirm that specific activity of the synthase depends upon enzyme concentration. The pH activity curve for *A. eutrophus* PHA synthase purified from *T. ni* cells is shown in Figure 14. The curve shows a broad activity maximum centered around pH 8.5. This result agrees well with prior work on the *A. eutrophus* PHB synthase although it is significantly different than results obtained for the PHB synthase from Z. *ramigera* for which the optimum was determined to be pH 7.0.

The effect of varying enzyme concentration in the presence of a fixed amount of substrate revealed an intriguing trend (Figure 15). From these data it appears that the extent of polymerization is dependent on the amount of enzyme included in the reaction mixture. This could be explained if there is a "terminal length" limitation of the polymer, which, once reached, can not be extended any further. If this is the case, it would also suggest that termination of the polymerization reaction, the release of the synthase from the polymer, and/or reinitiation of polymerization by the newly released synthase are relatively slow events since no evidence of these reactions are seen within the timecourse of these studies. The phenomenon observed in Figure 15 is not the result of

decay of the enzyme over the course of the assay since virtually identical results are obtained following a 10 minute preincubation of the synthase at 25°C.

It must also be noted that comparisons of the direct spectrophotometric assays used here and the more common assay involving the use of Ellman's reagent, DTNB, (Ellman, *supra*) in the formation of thiolate of coenzyme-A showed that the values determined by the direct method were approximately 70% of the values determined using Ellman's reagent. This may be due to phase separation occurring in the cuvettes as the relatively insoluble polymer is formed. In support of this notion, a faint haze or opalescence in the cuvette developed during the course of the reaction, particularly at higher substrate concentrations.

PHA synthase purified from insect cells appears to be relatively stable. Examination of activity following storage, in liquid $N_2$ and at -20°C in the presence of 50% glycerol showed that approximately 50% of synthase activity remained after 7 weeks when stored in liquid $N_2$ and approximately 75% of synthase activity remained after 7 weeks when stored at -20°C in the presence of 50% glycerol.

The expression of PHA synthase from *A. eutrophus* in a baculovirus expression system results in the synthase constituting approximately 50% of total protein 60 hours post-infection; however, approximately 50-75% of the synthase is observed in the membrane-associated fraction. This elevated level of expression allowed purification of the soluble PHA synthase using a single chromatographic step on HA. The purity of this preparation is estimated to be approximately 90% (intact PHA synthase and 3 proteolysis products).

The initial specific activity of 12 U/mg was approximately 20-fold higher than the most successful previous

efforts at overexpression of *A. eutrophus* PHA synthase. The synthase reported here was isolated from a 250 ml culture with 70% recovery which represents an improvement of 500-fold (1000 U / 64 U x 8 L / 0.25 L) when compared to an 8 L *E. coli* culture with 40% recovery. This high expression level should provide sufficient PHA synthase for extensive structural, functional, and mechanistic studies. Furthermore, it is clear that the baculovirus expression system is an attractive option for isolation of other PHA synthases from various sources.

PHA synthase produced in the baculovirus system was of sufficient potency to allow direct spectrophotometric analysis of the hydrolysis of the thioester bond of HBCoA at 232 nm. These assays revealed a lag period of approximately 60 seconds, the length of which was variable and inversely related to enzyme concentration. Such a lag period presumably reflects a slow step in the reaction, perhaps correlating to dimerization of the enzyme, the priming, and/or initiation steps in formation of PHB. Size exclusion chromatographic examination of the PHB synthase native MW indicated two forms of the synthase. One form showed a MW of approximately 100-160 kDa and the other showed a MW of approximately 50-80 kDa; these two forms likely represent the dimer and monomer of PHA synthase, respectively. Similar results have been reported previously in which two forms of approximately 60 and 130 kDa were observed. Comparisons of the direct assay reported here and the indirect assay using DTNB revealed that the former resulted in values that were 70% of the values determined by the DTNB indirect assay. Although the reason for this difference has not been examined in detail, it is probable that the apparent phase separation that occurred upon PHB formation in the short pathlength cuvettes used, particularly with high [HBCoA], results in this discrepancy.

Enzymatic analyses of the PHA synthase have found that the enzyme has a broad pH optimum centered at pH 8.5; however, the studies described herein have been performed at pH 7.2 to provide comparative values with the results of others. Moreover, the specific activity of this enzyme is dependent upon enzyme concentration which confirms and extends earlier results (Gerngross et al., *supra*).

In studies intended to examine the dependence of activity upon enzyme concentration, it became apparent that the extent of the polymerization reaction is dependent on the amount of enzyme included in the reaction mixture. Specifically, decreasing the amount of enzyme leads not only to decreased velocity of reaction but also to a decreased extent of condensation (Figure 15). One possible explanation is that the enzyme is thermally labile; however, identical assays in which the enzyme is preincubated at 25°C for 10 minutes prior to initiation of the reaction had similar results. Another possibility is that a terminal-length of the polymer is reached precluding further condensations until the particular synthase molecule is released from the terminal-length polymer.

This work clearly demonstrates the value of the baculovirus expression system for the production of *A. eutrophus* PHA synthase and for the potential application to studies of other PHA synthases. Furthermore, the high level of expression obtained using the baculoviral system should allow convenient analysis for substrate-specificity and structure-function studies of PHA synthases from relatively crude insect cell extracts.

## Example 2

Co-expression of rat FAS dehydrase mutant cDNA and PHB synthase gene in insect cells.

Expression of a rat FAS DH- cDNA in *Sf9* cells has been reported previously (Rangan et al., J. Biol. Chem., 266, 19180 (1991);

Joshi et al., Biochem. J., 296, 143 (1993)). Once activity of the *phbC* gene product had been established in insect cells (see Example 1), baculovirus clones containing the rat FAS DH- cDNA and BacPAK6::*phbC* were employed in a double infection strategy to determine if PHB would be produced in insect cells. It was not known if an intracellular pool of R(-)-3-hydroxybutyrate would be stable or available as a substrate for the PHB synthase. In order for the R-(-)-3-hydroxybutyrylCoA to be available as a substrate, the R-(-)-3-hydroxybutyrylCoA released from rat FAS DH- protein must be trapped by the PHB synthase and incorporated into a polymer at a rate faster than β-oxidation, which would regenerate acetylCoA. It was also not known if the stereochemical configuration of the 3-hydroxyl group, which must be in the R form, would be recognized as a substrate by PHB synthase. Fortunately, previous biochemical studies on eukaryotic FASs indicated that the R form of 3-hydroxylbutyrylCoA would be generated (Wakil et al., J. Biol. Chem., 237, 687 (1962)).

SDS-PAGE of protein samples from a time course of uninfected, single-infected, and dual-infected S*f*21 cells was performed (Figure 16). From these data, it is clear that the rat FAS DH mutant and PHB synthase polypeptides are efficiently co-expressed in S*f*21 cells. However, co-expression results in ~50% reduced levels of both polypeptides compared to S*f*21 cells that are producing the individual proteins. Western analysis using anti-rat FAS (Rangan et al., *supra*) and anti-PHA synthase antibodies confirmed simultaneous production of the corresponding proteins.

To provide further evidence that PHB was being synthesized in insect cells, *T. ni* cells which had been infected with a baculovirus vector encoding rat FAS $DH^0$ and/or a baculovirus vector encoding PHA synthase were analyzed for the presence of granules.

Infected cells were fixed in paraformaldehyde and incubated with anti-PHA synthase antibodies (Williams et al., Protein Exp. Purif., 7, 203 (1996)). Granules were observed only in doubly infected cells (Williams et al., App. Environ. Micro., 62, 2540 (1996)).

Characterization of PHB production in insect cells. In order to determine if *de novo* synthesis of PHB was occurring in S*f*21 cells that co-express the rat FAS DH mutant and PHB synthase, fractions of these samples were extracted, the extract subjected to propanolysis, and analyzed for the presence of propylhydroxybutyrate by gas chromatography (Figure 17). A unique peak with a retention time that coincided with a propylhydroxybutyrate standard was detected only in the double infection samples at 48 and 72 hours, in contrast to the individually expressed gene products and uninfected controls, which were negative. These samples were analyzed further by GC/MS to confirm the identity of the product. Figure 18 shows mass spectroscopy data corresponding to the material obtained from peak 10.1 in the gas chromatograph compared to an propylhydroxybutyrate standard. The results show that PHB synthesis is occurring only in S*f*21 cells co-expressing the rat FAS DH mutant cDNA and the *phbC* gene from *A. eutrophus*. Integration of the peak in the gas chromatograph corresponding to propylhydroxybutyrate revealed that approximately 1 mg of PHB was isolated from 1 liter culture of S*f*21 cells (approximately 600 mg dry cell weight of S*f*21 cells). Thus, the ratFAS206 protein effectively replaces the β-ketothiolase and acetoacetyl-CoA reductase functions, resulting in the production of PHB by a novel pathway.

The approach described here provides a new strategy to combine metabolic pathways that are normally engaged in primary anabolic functions for production of polyesters. The premature termination of the normal fatty acid biosynthetic pathway to provide

suitably modified acylCoA monomers for use in PHA synthesis can be applied to both prokaryotic and eukaryotic expression since the formation of polymer will not be dependent on specialized feedstocks. Thus, once a recombinant PHA monomer synthase is introduced into a prokaryotic or eukaryotic system, and co-expressed with the appropriate PHA synthase, novel biopolymer formation can occur.

**Example 3**

Cloning and Sequencing of the *vep* ORFI PKS Gene Cluster

The entire PKS cluster from *Streptomyces venezuelae* was cloned using a heterologous hybridization strategy. A 1.2 kb DNA fragment that hybridized strongly to a DNA encoding an *ery*A PKS β-ketoacyl synthase domain was cloned and used to generate a plasmid for gene disruption. This method generated a mutant strain blocked in the synthesis of the antibiotic. A *S. venezuelae* genomic DNA library was generated. and used to clone a cosmid containing the complete methymycin aglycone PKS DNA. Fine-mapping analysis was performed to identify the order and sequence of catalytic domains along the multifunctional PKS (Figure 19). DNA sequence analysis of the *vep* ORFI showed that the order of catalytic domains is KSQ/AT/ACP/KS/AT/KR/ACP/KS/AT/DH/KR/ACP. The complete DNA sequence, and corresponding amino acid sequence, of the *vep* ORFI is shown in Figure 23 (SEQ ID NO:1 and SEQ ID NO:2, respectively).

The sequence data indicated that the PKS gene cluster encodes a polyene of twelve carbons. The *vep* gene cluster contains 5 polyketide synthase modules, with a loading module at its 5′ end and an ending domain at its 3′ end. Each of the sequenced modules includes a keto-ACP (KS), an acyltransferase (AT), a dehydratase (DH),

a keto-reductase (KR), and an acyl carrier protein domain. The six acyltransferase domains in the cluster are responsible for the incorporation of six acetyl-CoA moieties into the product. The loading module contains a KSQ, an AT and an ACP domain. KSQ refers to a domain that is homologous to a KS domain except that the active site cysteine (C) is replaced by glutamine (Q). There is no counterpart to the KSQ domain in the PKS clusters which have been previously characterized.

The ending domain (ED) is an enzyme which is responsible for the attachment of the nascent polyketide chain onto another molecule. The amino acid sequence of ED resembles an enzyme, HetM, which is involved in *Anabaena* heterocyst formation. The homology between *vep* and HetM suggests that the polypeptide encoded by the *vep* gene cluster may synthesize a polyene-containing composition which is present in the spore coat or cell wall of its natural host, *S. venezuelae*.

**Example 4**

To provide a recombinant monomer synthase that generates a saturated β-hydroxyhexanoylCoA or unsaturated β-hydroxyhexanoylCoA monomer, the linear correspondence between the genetic organization of the Type I macrolide PKS and the catalytic domain organization in the multifunctional proteins is assessed (Donadio et al., *supra*, 1991; Katz et al., Ann. Rev. Microbiol., 47, 875 (1993)). First, a DNA encoding a TE is added to the 3' end of an ORFI of a Type I PKS, preferably the *met* ORF I (Figure 6) as recently described by Cortes et al. (Science, 268, 1487 (1995) in the erythromycin system. To ensure that the DNA encoding the TE is completely active, DNA encoding a linker region separating a normal ACP-TE region in a PKS, for example the one found in *met* PKS ORF5 (Figure

5), will be incorporated into the DNA. The resulting vector can be introduced into a host cell and the TE activity, rate of release of the CoA product, and identity of the fatty acid chain determined.

The acyl chain that is most likely to be released is the CoA ester, specifically the 3-hydroxy-4-methyl heptenoylCoA ester, since the fully elongated chain is presumably released in this form prior to macrolide cyclization. If the CoA form of the acyl chain is not observed, then a gene encoding a CoA ligase will be cloned and co-expressed in the host cell to catalyze formation of the desired intermediate.

There is clear precedent for release of the predicted premature termination products from mutant strains of macrolide-producing *Streptomyces* that produce intermediates in macrolide synthesis (Huber et al., Antimicrob. Agents Chemother., 34, 1535 (1990); Kinoshita et al., J. Chem. Soc., Chem. Comm., 14, 943 (1988)). The structure of these intermediates is consistent with the linear organization of functional domains in macrolide PKSs, particularly those related to *eryA*, *tyl*, and *met*. Other known PKS gene clusters include, but are not limited to, the gene cluster encoding 6-methysalicylic acid synthase (Beck et al., Eur. J. Biochem., 192, 487 (1990)), soraphen A (Schupp et al., J. Bacteriol., 177, 3673 (1995), and sterigmatocystin (Yu et al., J. Bacteriol., 177, 4792 (1995)).

Once the release of the 3-hydroxy-4-methyl heptenoylCoA ester is established, DNA encoding the extender unit AT in *met* module 1 is replaced to change the specificity from methylmalonylCoA to malonylCoA (Figures 4-6). This change eliminates methyl group branching in the β-hydroxy acyl chain. While comparison of known AT amino acid sequences shows high overall amino acid sequence conservation, distinct regions are readily apparent where significant deletions or insertions have occurred. For

example, comparison of malonyl and methylmalonyl amino acid sequences reveals a 37 amino acid deletion in the central region of the malonyltransferase. Thus, to change the specificity of the methylmalonyl transferase to malonyl transferase, the *met* ORFI DNA encoding the 37 amino acid sequence of MMT will be deleted, and the resulting gene will be tested in a host cell for production of the desmethyl species, 3-hydroxyheptenoylCoA. Alternatively, the DNA encoding the entire MMT can be replaced with a DNA encoding an intact MT to affect the desired chain construction.

After replacing MMT with MT, DNA encoding DH/ER will be introduced into DNA encoding *met* ORFI module 1. This modification results in a multifunctional protein that generates a methylene group at C-3 of the acyl chain (Figure 6). The DNA encoding DH/ER will be PCR amplified from the available *eryA* or *tyl* PKS sequences, including the DNA encoding the required linker regions, employing a primer pair to conserved sequences 5' and 3' of the DNA encoding DH/ER. The PCR fragment will then be cloned into the *met* ORFI. The result is a DNA encoding a multifunctional protein (MT* DH/ER*TE*). This protein possesses the full complement of keto group processing steps and results in the production of heptenoylCoA.

The DNA encoding dehydrase in *met* module 2 is then inactivated, using site-directed mutagenesis in a scheme similar to that used to generate the rat FAS DH- described above (Joshi et al., J. Biol. Chem., 268, 22508 (1993)). This preserves the required (R)-3-hydroxy group which serves as the substrate for PHA synthases and results in a (R)-3-hydroxyheptanoylCoA species.

The final domain replacement will involve the DNA encoding the starter unit acyltransferase in *met* module 1 (Figure 5), to change the specificity from propionyl CoA to acetyl CoA. This shortens the (R)-3-hydroxy acyl chain from heptanoyl to hexanoyl.

The DNA encoding the catalytic domain will need to be generated based on a FAS or 6-methylsalicylic acid synthase model (Beck et al., Eur. J. Biochem., 192, 487 (1990)) or by using site-directed mutagenesis to alter the specificity of the resident *met* PKS propionyltransferase sequence. Limiting the initiator species to acetylCoA can result in the use of this starter unit by the monomer synthase. Previous work with macrolide synthases have shown that some are able to accept a wide range of starter unit carboxylic acids. This is particularly well documented for avermectin synthase, where over 60 new compounds have been produced by altering the starter unit substrate in precursor feeding studies (Dutton et al., J. Antibiotics, 44, 357 (1991)).

**Example 5**

To provide a recombinant monomer synthase that synthesizes 3-hydroxyl-4-hexenoic acid, a precursor for polyhydoxyhexenoate, the DNA segment encoding the loading and the first module of the *vep* gene cluster was linked to the DNA segment encoding module 7 of the *tyl* gene cluster so as to yield a recombinant DNA molecule encoding a fusion polypeptide which has no amino acid diffences relative to the corresponding amino acid sequence of the parent modules. The fusion polypeptide catalyzes the synthesis of 3-hydroxyl-4-hexenoic acid. The recombinant DNA molecule was introduced into SCP2, a *Streptomyces* vector, under the control of the *act* promoter (pDHS502, Figure 20). A polyhydroxyalkanoate polymerase gene, *pha*C1 from *Pseudomonas oleavorans*, was then introduced downstream of the recombinant PKS cluster (pDHS505; Figures 22 and 23). The DNA segment encoding the polyhydroxyalkanoate polymerase is linked to the DNA segment encoding the recombinant PKS synthase so as to yield a fusion polypeptide which synthesizes polyhydroxyhexenoate in *Streptomyces*. Polyhydroxyhexenoate, a biodegradable thermoplastic,

is not naturally synthesized in *Streptomyces*. or as a major product in any other organism. Moreover, the unsaturated double bond in the side chain of polyhydroxyhexenoate may result in a polymer which has superior physical properties as a biodegradable thermoplastic over the known polyhydroxyalkanoates.

The complete disclosure of all patents, patent documents and publications cited herein are incorporated herein by reference as if individually incorporated. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described for variations obvious to one skilled in the art will be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. A baculovirus expression cassette comprising a nucleic acid molecule encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in an insect cell.

2. The expression cassette of claim 1 wherein the source of the nucleic acid molecule is a bacterium.

3. The expression cassette of claim 2 wherein the bacterium is *Alcaligenes eutrophus*.

4. An expression cassette comprising a nucleic acid molecule encoding a polyhydroxyalkanoate monomer synthase operably linked to a promoter functional in a host cell, wherein the nucleic acid molecule comprises a first DNA segment encoding a first module and a second DNA segment encoding a second module, wherein the DNA segments together encode a polyhydroxyalkanoate monomer synthase, and wherein no more than one DNA segment is derived from the *eryA* gene cluster of *Saccharopolyspora erythraea*.

5. The expression cassette of claim 4 wherein the source of at least one DNA segment is bacterial DNA.

6. A method of providing a polyhydroxyalkanoate synthase, comprising:

(a) introducing an expression cassette comprising a DNA molecule encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in a eukaryotic

cell into the eukaryotic cell, wherein the eukaryotic cell is not of plant origin; and

(b) expressing the DNA molecule encoding the polyhydroxyalkanoate synthase in the eukaryotic cell.

7. The method of claim 6 wherein the polyhydroxyalkanoate synthase is polyhydroxybutyrate synthase.

8. The method of claim 6 wherein the polyhydroxyalkanoate synthase is derived from a bacterium.

9. The method of claim 8 wherein the bacterium is *Alcaligenes eutrophus*.

10. The method of claim 6 wherein the eukaryotic cell is of insect origin.

11. The method of claim 10 wherein the expression cassette is a baculovirus expression cassette.

12. The method of claim 6 further comprising isolating polyhydroxyalkanoate synthase from the eukaryotic cell.

13. A method of providing a polyhydroxyalkanoate polymer, comprising:

(a) introducing into a eukaryotic cell (i) a first expression cassette comprising a DNA segment encoding a fatty acid synthase in which the dehydrase activity is inactivated that is operably linked to a promoter functional in the eukaryotic cell, and (ii) a second expression cassette comprising a DNA segment encoding a

polyhydroxyalkanoate synthase operably linked to a promoter functional in the eukaryotic cell; and

(b) expressing the DNA segments so as to yield a polyhydroxyalkanoate polymer in the eukaryotic cell.

14. The method of claim 13 wherein the eukaryotic cell is of insect origin.

15. The method of claim 13 wherein the dehydrase activity is inactivated by mutating the catalytic site.

16. The method of claim 13 wherein the fatty acid synthase is a rat fatty acid synthase.

17. The method of claim 13 wherein the polyhydroxyalkanoate synthase is a polyhydroxybutyrate synthase.

18. The method of claim 13 wherein the fatty acid synthase produces a premature termination product.

19. The method of claim 13 wherein the fatty acid synthase catalyzes the synthesis of D(-)-3-hydroxybutyrate in the eukaryotic cell.

20. The method of claim 13 wherein the polyhydroxyalkanoate polymer is polyhydroxybutyrate.

21. The method of claim 13 wherein the first and second expression cassettes are on different DNA molecules.

22. An isolated and purified DNA molecule comprising a first DNA segment encoding a first module and a second DNA segment encoding a second module, wherein the DNA segments together encode a recombinant polyhydroxyalkanoate monomer synthase, and wherein no more than one DNA segment is derived from the *eryA* gene cluster of *Saccharopolyspora erythraea*.

23. The isolated DNA molecule of claim 22 wherein the first DNA segment is derived from the *vep* gene cluster of *Streptomyces. venezuelae*.

24. The isolated DNA molecule of claim 22 wherein the second DNA segment is derived from the *tyl* gene cluster of *Streptomyces*.

25. The isolated DNA molecule of claim 22 wherein the second DNA segment comprises a DNA encoding a thioesterase which is located at the 3' end of the second DNA segment.

26. The isolated DNA molecule of claim 25 wherein the second DNA segment comprises a DNA encoding an acyl carrier protein which is located 5' to the DNA encoding the thioesterase.

27. The isolated DNA molecule of claim 26 wherein the second DNA segment comprises a DNA encoding a linker region, wherein the DNA encoding the linker region is located between the DNA encoding the acyl carrier protein and the DNA encoding the thioesterase.

28. The isolated DNA molecule of claim 22 wherein the first DNA segment comprises DNA encoding two acyl transferases, wherein the DNA encoding the first acyl transferase is 5' to the DNA encoding the second acyl transferase.

29. The isolated DNA molecule of claim 28 wherein the second acyl transferase adds acyl groups to malonylCoA.

30. The isolated DNA molecule of claim 22 wherein the first DNA segment comprises a DNA encoding a dehydrase.

31. The isolated DNA molecule of claim 22 wherein the first DNA segment comprises a DNA encoding a dehydrase and an enoyl reductase.

32. The isolated DNA molecule of claim 22 wherein the second DNA segment comprises a DNA encoding an inactive dehydrase.

33. The isolated DNA molecule of claim 22 wherein the first DNA segment comprises a DNA encoding an acyl transferase.

34. The isolated DNA molecule of claim 33 wherein the acyl transferase domain binds an acyl CoA substrate.

35. The isolated DNA molecule of claim 22 comprising a first DNA segment encoding a first module and a second DNA segment encoding a second module, wherein the DNA segments together encode a recombinant polyhydroxyalkanoate monomer synthase, and wherein no more than one DNA

segment is derived from the *ery*A gene cluster of *Saccharopolyspora erythraea*.

36. The isolated DNA molecule of claim 35 wherein the first DNA segment encodes the first module from the *vep* gene cluster and the second DNA segment encodes module 7 from the *tyl* gene cluster.

37. A method of providing a polyhydroxyalkanoate monomer, comprising:

(a) introducing into a host cell a DNA molecule comprising a DNA segment encoding a recombinant polyhydroxyalkanoate monomer synthase operably linked to a promoter functional in the host cell, wherein the recombinant polyhydroxyalkanoate monomer synthase comprises a first module and a second module; and

(b) expressing the DNA encoding the recombinant polyhydroxyalkanoate monomer synthase in the host cell so as to generate a polyhydroxyalkanoate monomer.

38. A method of providing a polyhydroxyalkanoate polymer, comprising:

(a) introducing into a host cell a first DNA molecule comprising a DNA segment encoding a recombinant polyhydroxyalkanoate monomer synthase operably linked to a promoter functional in a host cell, wherein the recombinant polyhydroxyalkanoate monomer synthase comprises a first module and a second module;

(b) introducing into the host cell of step (a) a second DNA molecule comprising a DNA segment encoding a

polyhydroxyalkanoate synthase operably linked to a promoter functional in the host cell; and

(c) expressing the DNAs encoding the recombinant polyhydroxyalkanoate monomer synthase and polyhydroxyalkanoate synthase in the host cell so as to generate a polyhydroxyalkanoate polymer.

39. The method of claim 37 or 38 wherein the first DNA segment encodes the first module from the *vep* gene cluster and the second DNA segment encodes module 7 from the *tyl* P gene cluster.

40. An isolated and purified DNA molecule comprising a first DNA segment encoding a fatty acid synthase and a second DNA segment encoding a module of a polyketide synthase.

41. The isolated DNA molecule of claim 40 wherein the second DNA segment encodes a β-ketoacyl synthase amino-terminal to an acyltransferase which is amino-terminal to a ketoreductase which is amino-terminal to an acyl carrier protein which is amino- terminal to a thioesterase.

42. The isolated DNA molecule of claim 40 wherein the second DNA segment is 3' to the DNA encoding the fatty acid synthase.

43. The isolated DNA molecule of claim 40 wherein the second DNA segment is separated from the first DNA segment by a DNA encoding a linker region.

44. The isolated DNA molecule of claim 41 wherein the DNA encoding the linker region is selected from the group consisting of *tyl* ORF1 $ACP_1$-$KS_2$, *tyl* ORF1 $ACP_2$-$KS_3$, *tyl* ORF3 $ACP_5$-$KS_6$, *ery*A ORF1 $ACP_1$-$KS_1$, *ery*A ORF1 $ACP_2$-$KS_2$, *ery*A ORF2 $ACP_3$-$KS_4$, and *ery*A ORF2 $ACP_5$-$KS_6$.

45. The isolated DNA molecule of claim 40 comprising a first DNA segment encoding a fatty acid synthase and a second DNA segment encoding a module of a polyketide synthase.

46. A method of providing a polyhydroxyalkanoate monomer, comprising:

(a) introducing into a host cell a DNA molecule comprising a first DNA segment encoding a fatty acid synthase and a second DNA segment encoding a polyketide synthase, wherein the first DNA segment is 5' to the second DNA segment, wherein the first DNA segment is operably linked to a promoter functional in the host cell, and wherein the first DNA segment is linked to the second DNA segment so that the linked DNA segments express a fusion protein; and

(b) expressing the DNA molecule in the host cell so as to generate a polyhydroxyalkanoate monomer.

47. The method of claim 46 wherein the host cell is selected from the group consisting of insect cells, *Streptomyces* cells and *Pseudomonas* cells.

48. The method of claim 46 wherein the DNA encoding the fatty acid synthase is eukaryotic in origin.

49. The method of claim 46 wherein the DNA encoding the fatty acid synthase is prokaryotic in origin.

50. The method of claim 46 wherein the DNA encoding the polyketide synthase module is derived from DNA encoding the *tyl* module F.

51. An expression cassette comprising a DNA molecule comprising a DNA segment encoding a fatty acid synthase and a polyhydroxyalkanoate synthase.

52. A method of providing a polyhydroxyalkanoate monomer synthase, comprising:

(a) introducing an expression cassette comprising a DNA molecule encoding a polyhydroxyalkanoate synthase operably linked to a promoter functional in a host cell, wherein the DNA comprises a first DNA segment encoding a first module and a second DNA segment encoding a second module wherein the DNA segments together encode a polyhydroxyalkanoate monomer synthase; and

(b) expressing the DNA molecule in the host cell.

53. An isolated and purified DNA molecule comprising a DNA segment encoding a *Streptomyces venezuelae* polyketide synthase.

54. The isolated DNA molecule of claim 53 wherein the DNA segment comprises SEQ ID NO:1.

55. The isolated DNA molecule of claim 53 wherein the DNA segment encodes a polypeptide having an amino acid sequence comprising SEQ ID NO:2.

56. The expression cassette of claim 4 wherein the first DNA segment encodes the first module from the *vep* gene cluster and the second DNA segment encodes module 7 from the *tyl* P gene cluster.

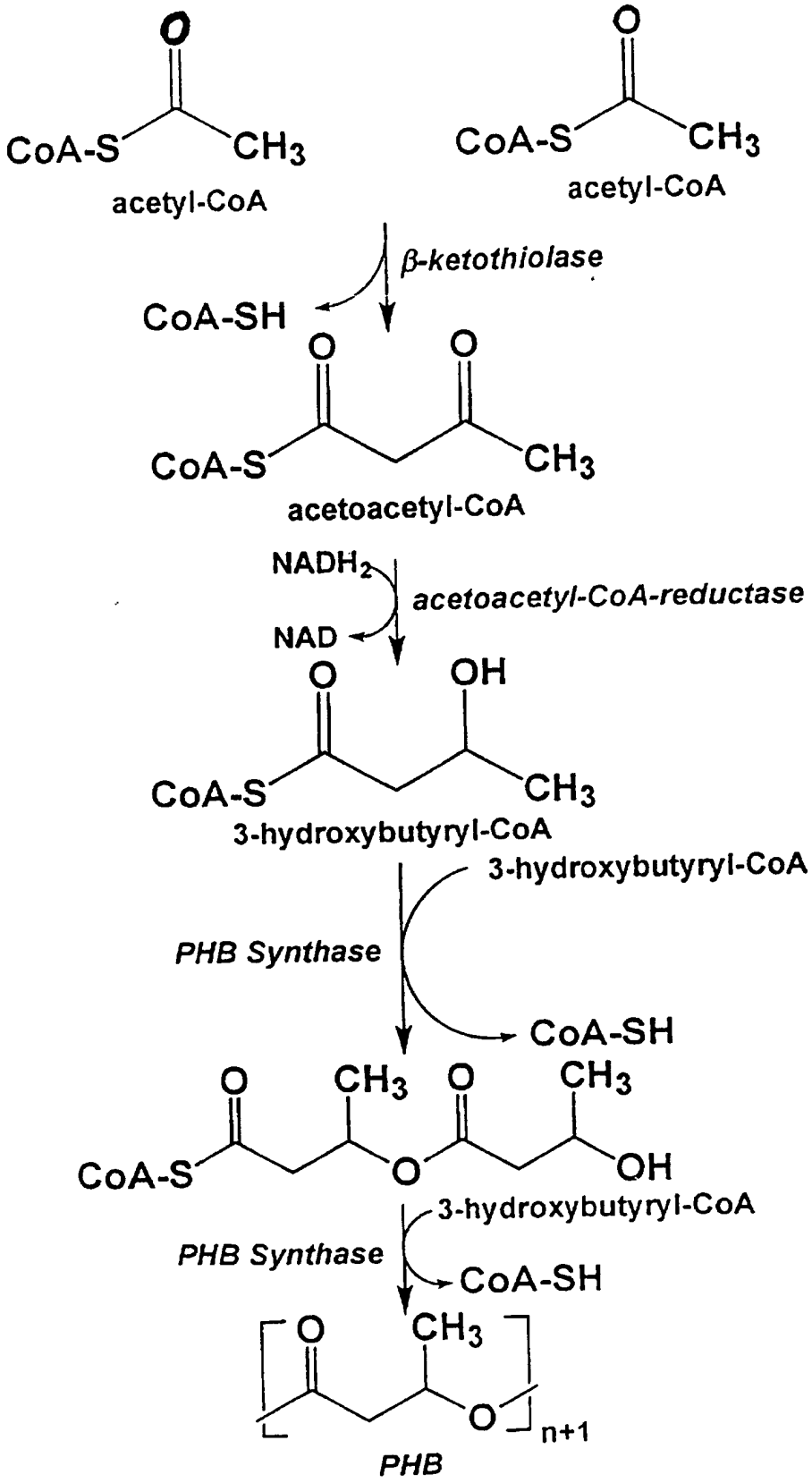
57. The expression cassette of claim 4 further comprising a third DNA segment encoding a polyhydroxyalkanoate synthase.

58. The method of claim 37 wherein the DNA molecule further comprises a DNA segment encoding a polyhydroxyalkanoate synthase.

59. The isolated DNA molecule of claim 22 or 36 further comprising a DNA segment encoding a polyhydroxyalkanoate synthase.

60. The method of claim 53 wherein the expression cassette further comprises a second DNA molecule encoding a polyhydroxyalkanoate synthase

Figure 1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acetyl-CoA
CoA-S
CH3
acetyl-CoA
β-ketothiolase
CoA-SH
CoA-S
CH3
acetoacetyl-CoA
NADH2
NAD
acetoacetyl-CoA-reductase
OH
CoA-S
CH3
3-hydroxybutyryl-CoA
3-hydroxybutyryl-CoA
PHB Synthase
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CH3
CH3
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CH3
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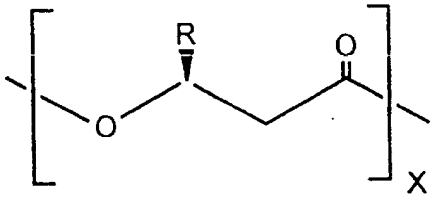
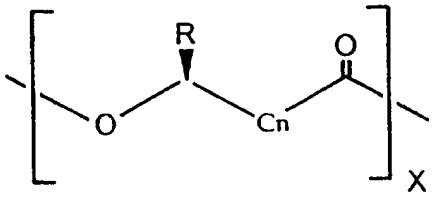
PHB




| R-group | Monomer | Abbreviation |
|---|---|---|
| methyl | 3-hydroxybutyrate | (3HB) |
| ethyl | 3-hydroxyvalerate | (3HV) |
| propyl | 3-hydroxycaproate | (3HC) |
| butyl | 3-hydroxyheptanoate | (3HH) |
| pentyl | 3-hydroxyoctanoate | (3HO) |
| hexyl | 3-hydroxynonanoate | (3HN) |
| heptyl | 3-hydroxydecanoate | (3HD) |
| octyl | 3-hydroxyundecanoate | (3HUD) |
| nonyl | 3-hydroxydodecanoate | (3HDD) |




| | |
|---|---|
| n = 1 | 3-hydroxyacyl monomer |
| n = 2 | 4-hydroxyacyl monomer |
| n = 3 | 5-hydroxyacyl monomer |

Figure 2

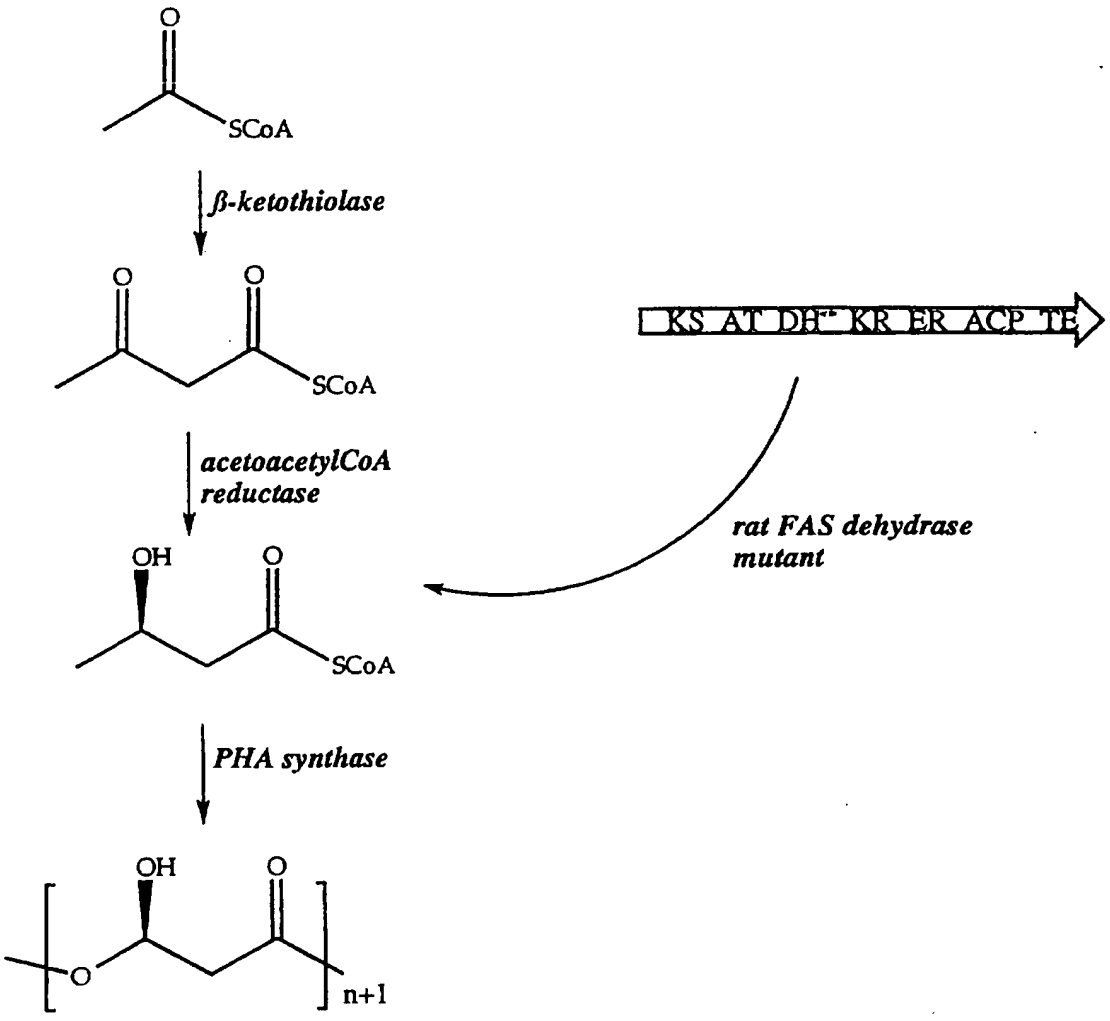
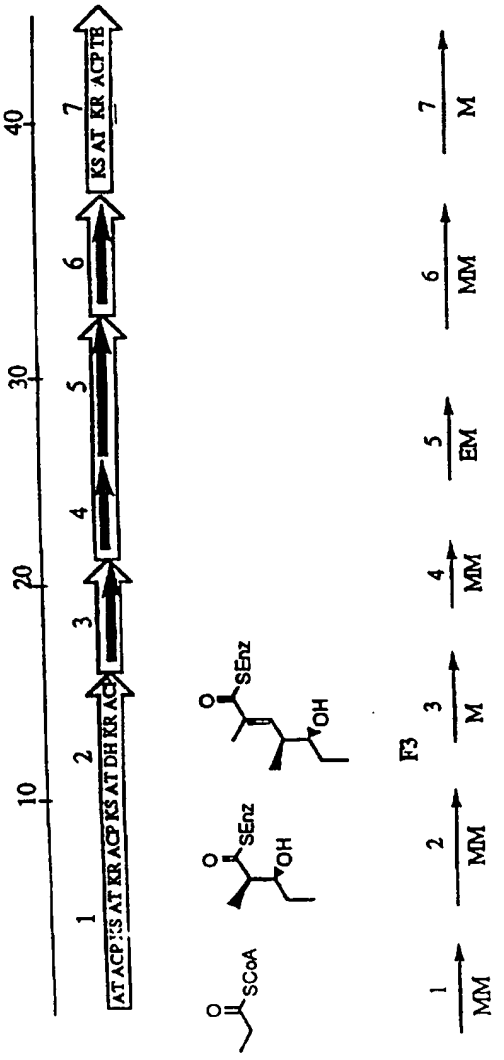

SCoA
ß-ketothiolase
SCoA
KS AT DH KR ER ACP TE
acetoacetylCoA reductase
rat FAS dehydrase mutant
OH
SCoA
PHA synthase
OH
n+1

Figure 3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20
30
40
1
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3
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6
7
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SCoA
SEnz
OH
SEnz
OH
F3
1
MM
2
MM
3
M
4
MM
5
EM
6
MM
7
M

Figure 4

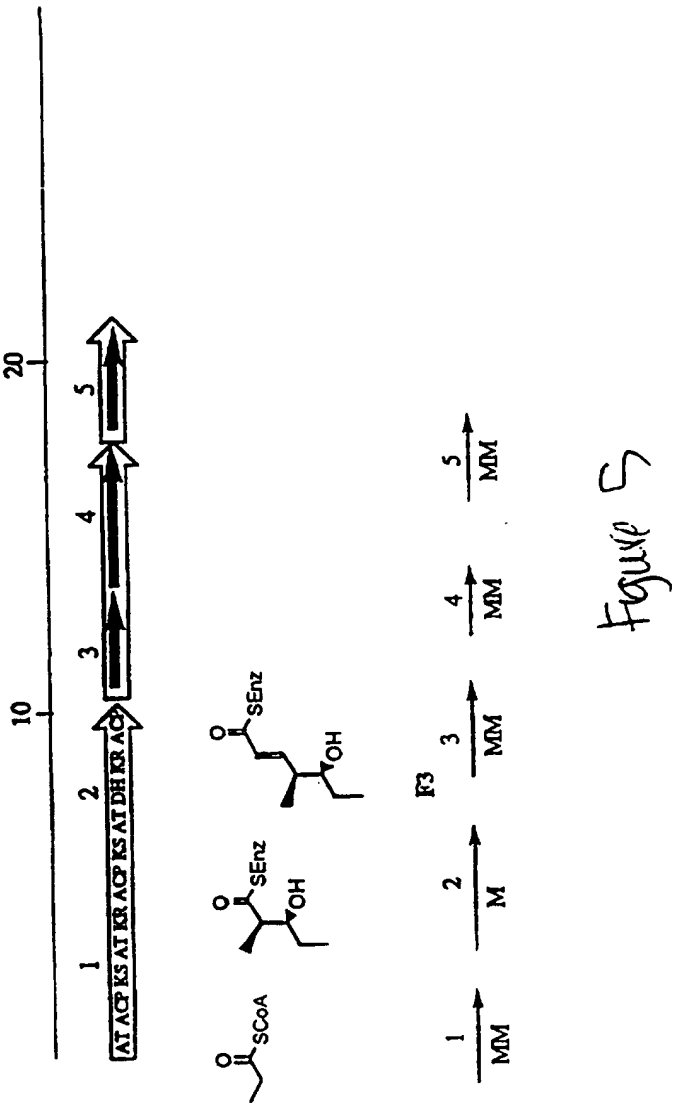

10
20
1
2
3
4
5
AT ACP KS AT KR ACP KS AT DH KR ACP
SCoA
SEnz
OH
F3
1
MM
2
M
3
MM
4
MM
5
MM

Figure 5

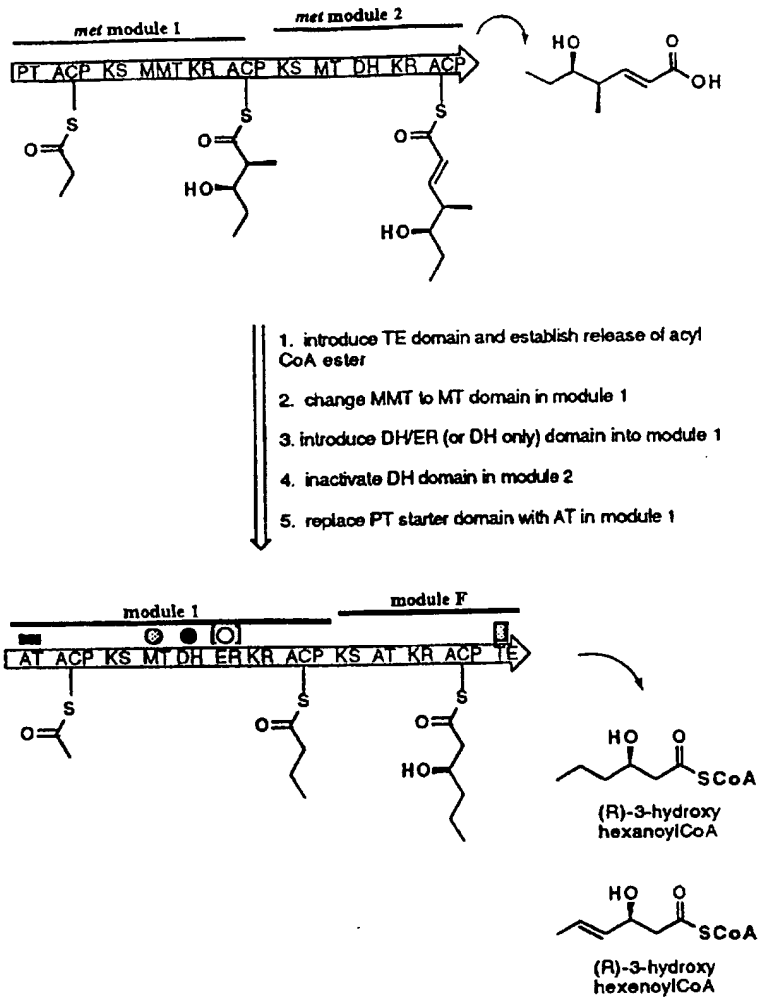

met module 1
met module 2
PT ACP KS MMT KR ACP KS MT DH KR ACP
HO
O
OH
1. introduce TE domain and establish release of acyl CoA ester
2. change MMT to MT domain in module 1
3. introduce DH/ER (or DH only) domain into module 1
4. inactivate DH domain in module 2
5. replace PT starter domain with AT in module 1
module 1
module F
AT ACP KS MT DH ER KR ACP KS AT KR ACP TE
SCoA
(R)-3-hydroxy hexanoylCoA
(R)-3-hydroxy hexenoylCoA

Fig. 6

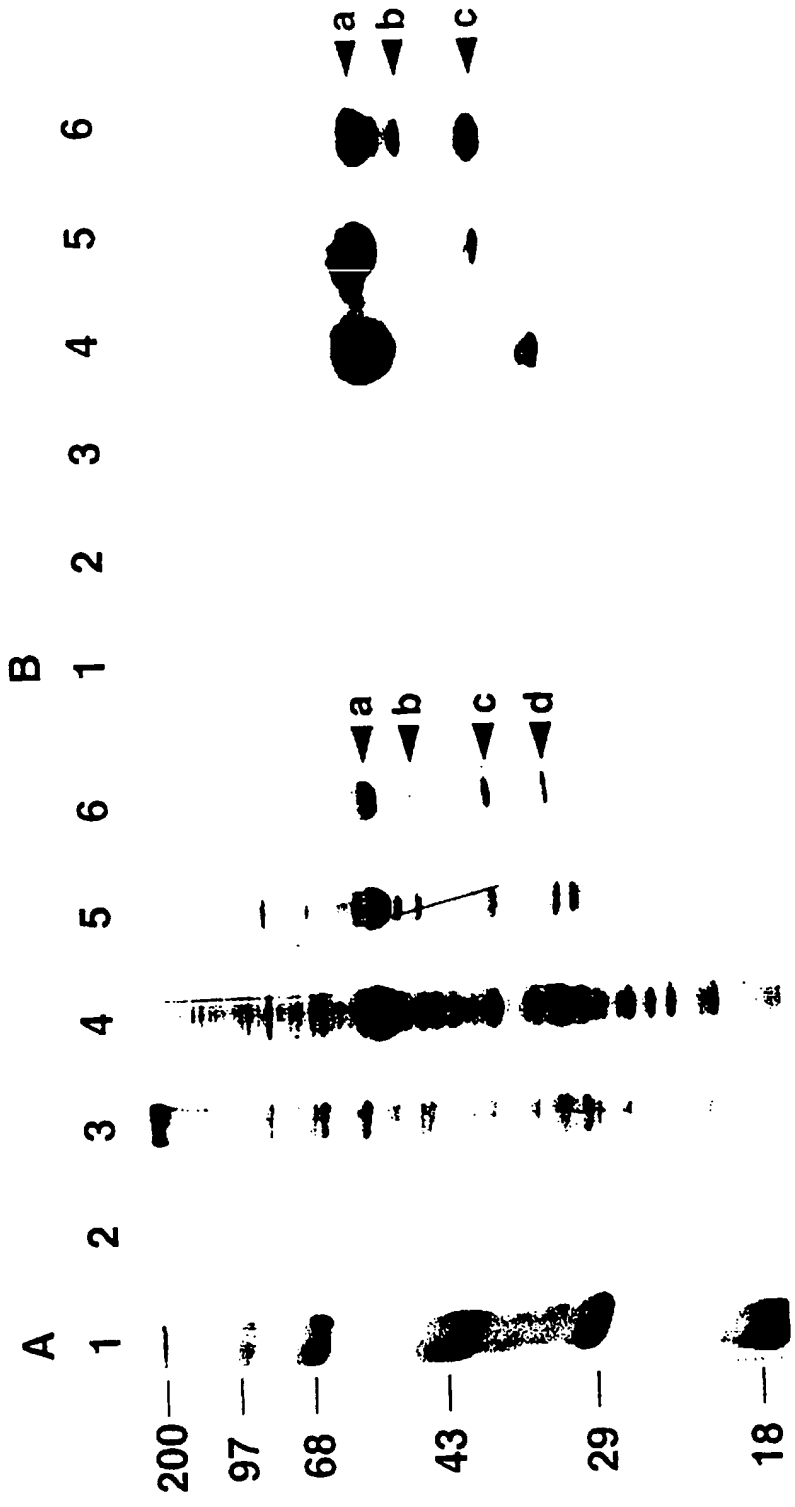



Figure 7

Figure 8

N-terminal sequence determined for PHA synthase

```
     1         10          20   25
a    MATGKGAAASTQEGKSQPFKVTPGP—
b          AAASTQEGKSQPFKVTPGP—
c             STQEGKSQPFKVTPGP—
```

Figure 9

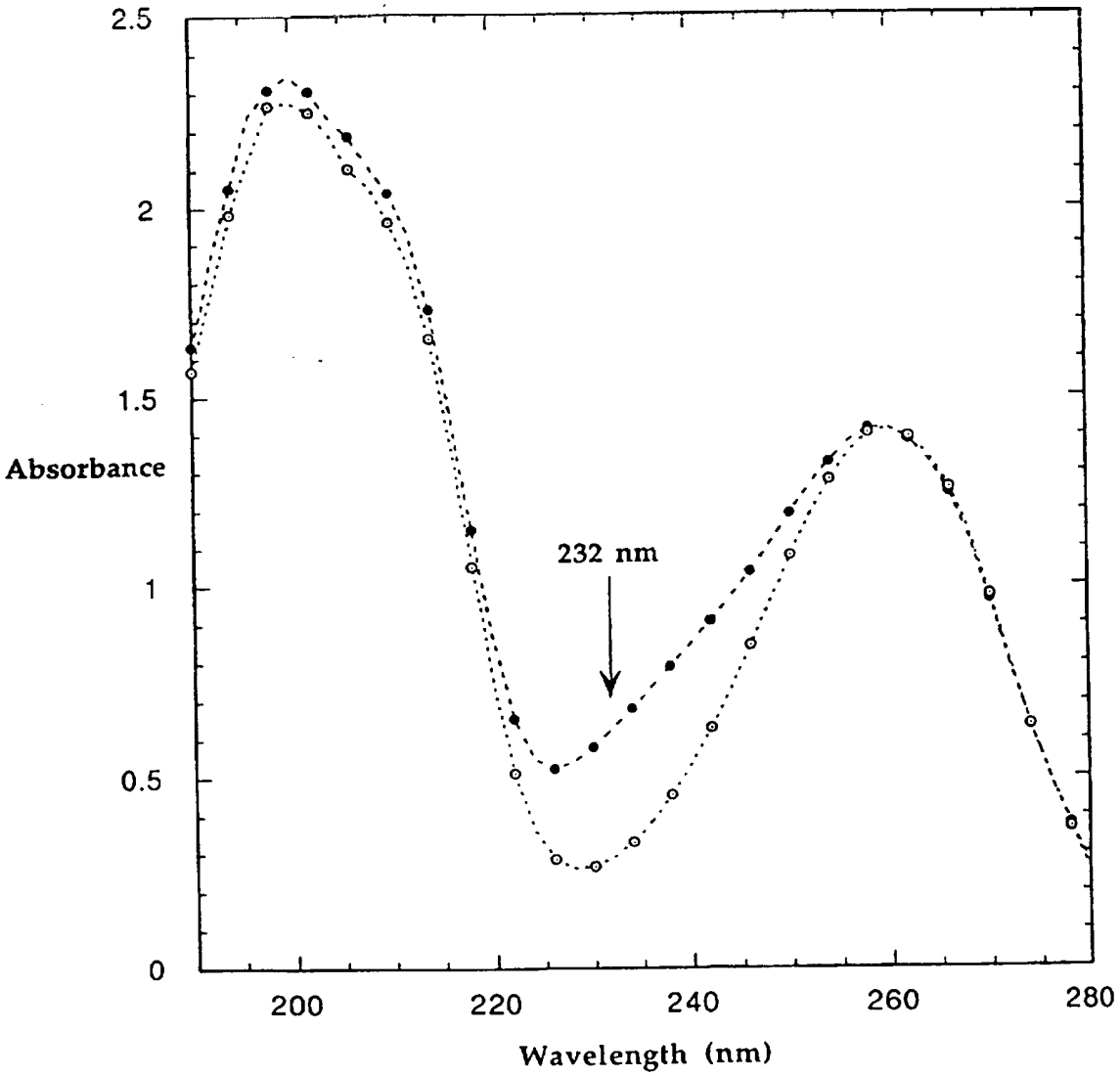

2.5
2
1.5
1
0.5
0
Absorbance
232 nm
200
220
240
260
280
Wavelength (nm)

Figure 10

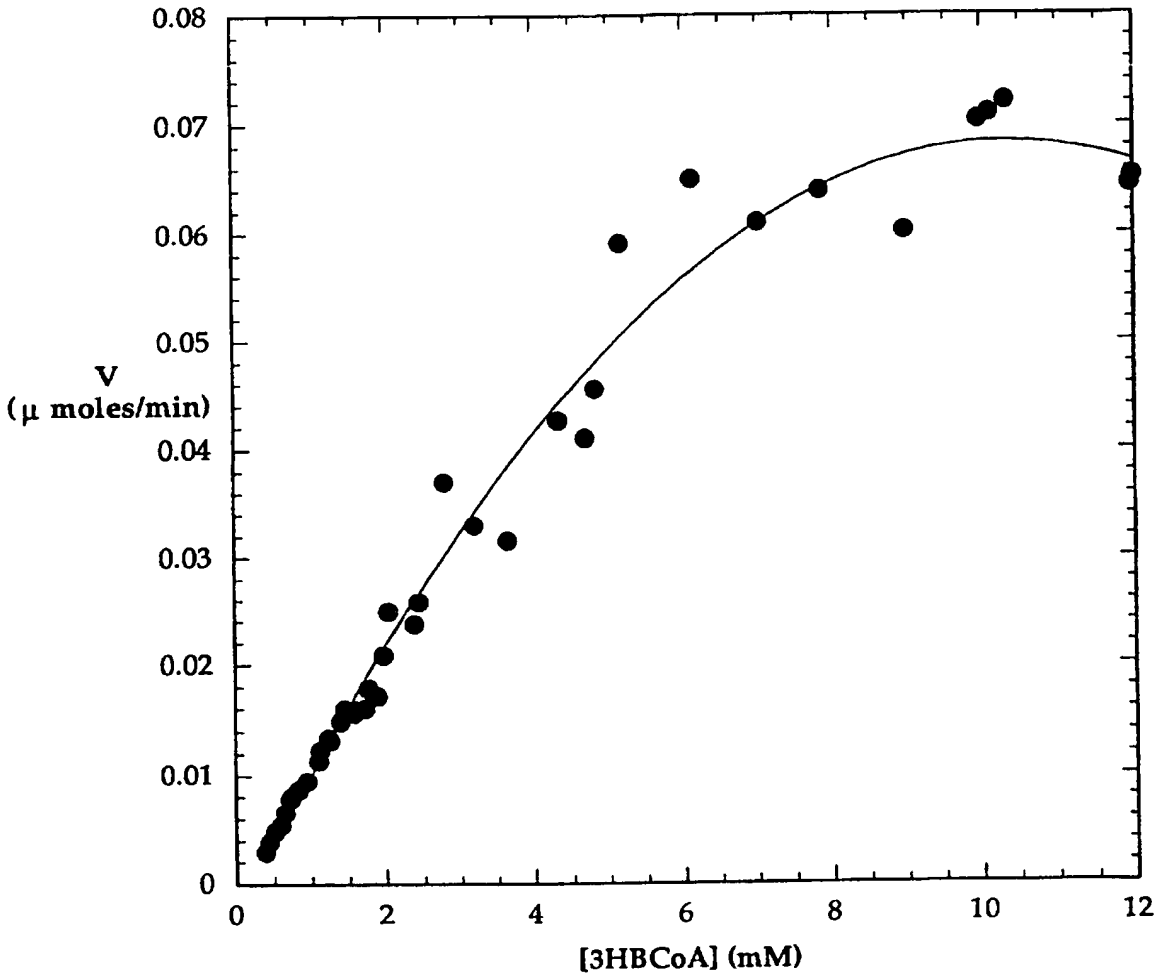

0.08
0.07
0.06
0.05
V
(μ moles/min)
0.04
0.03
0.02
0.01
0
0
2
4
6
8
10
12
[3HBCoA] (mM)

Figure 11

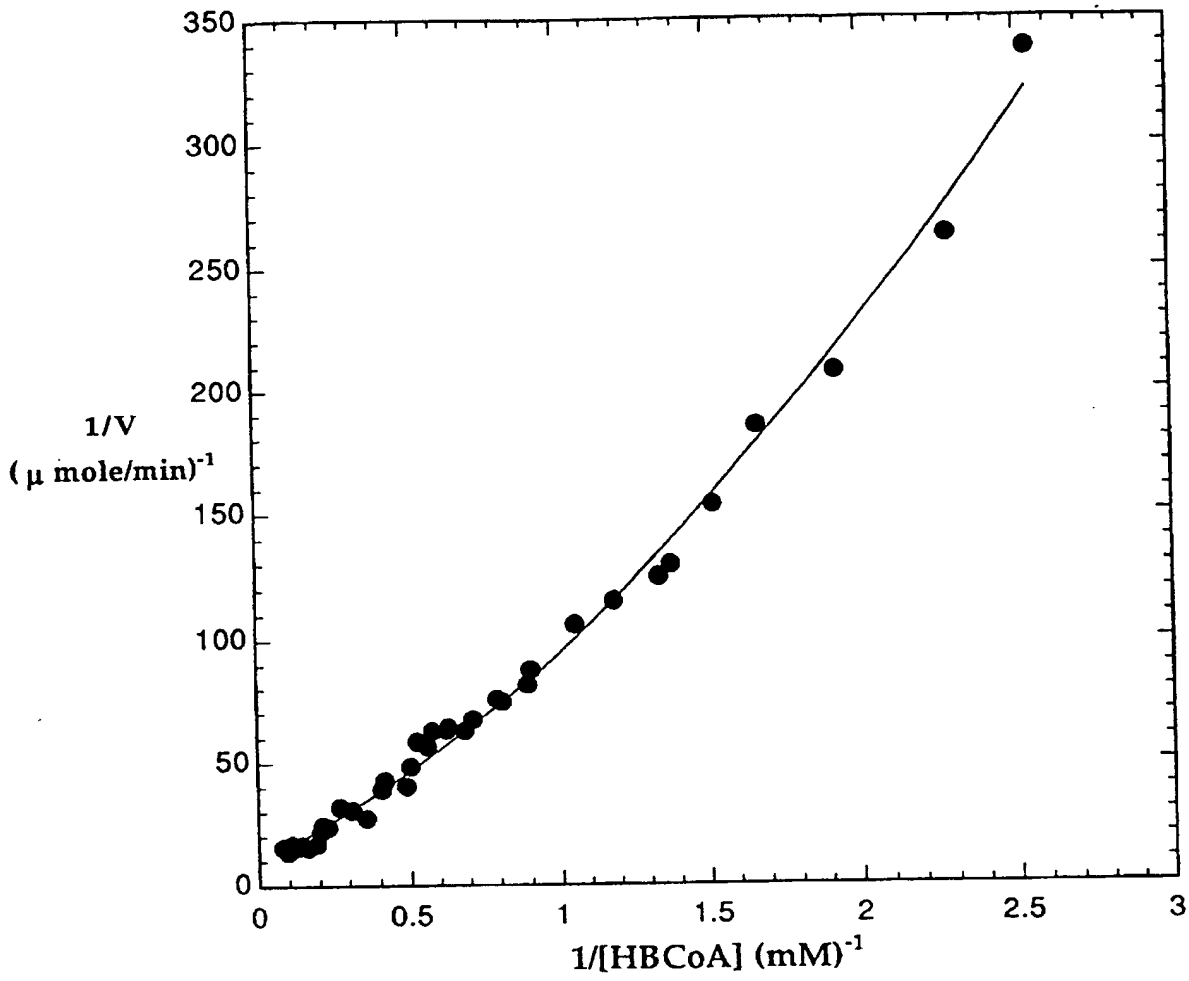

350
300
250
200
150
100
50
0
1/V
(μ mole/min)-1
0
0.5
1
1.5
2
2.5
3
1/[HBCoA] (mM)-1

Figure 17

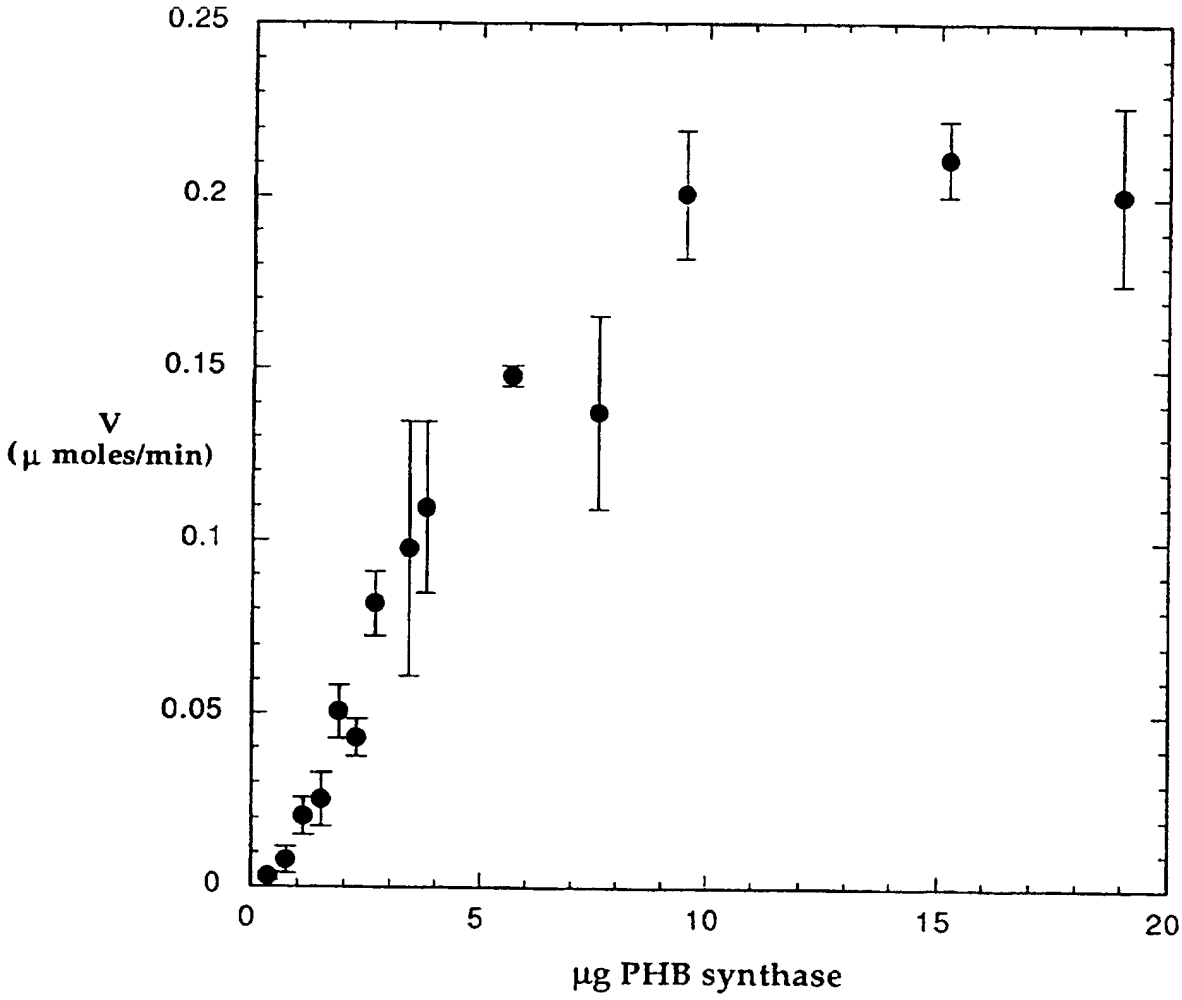


Figure 13

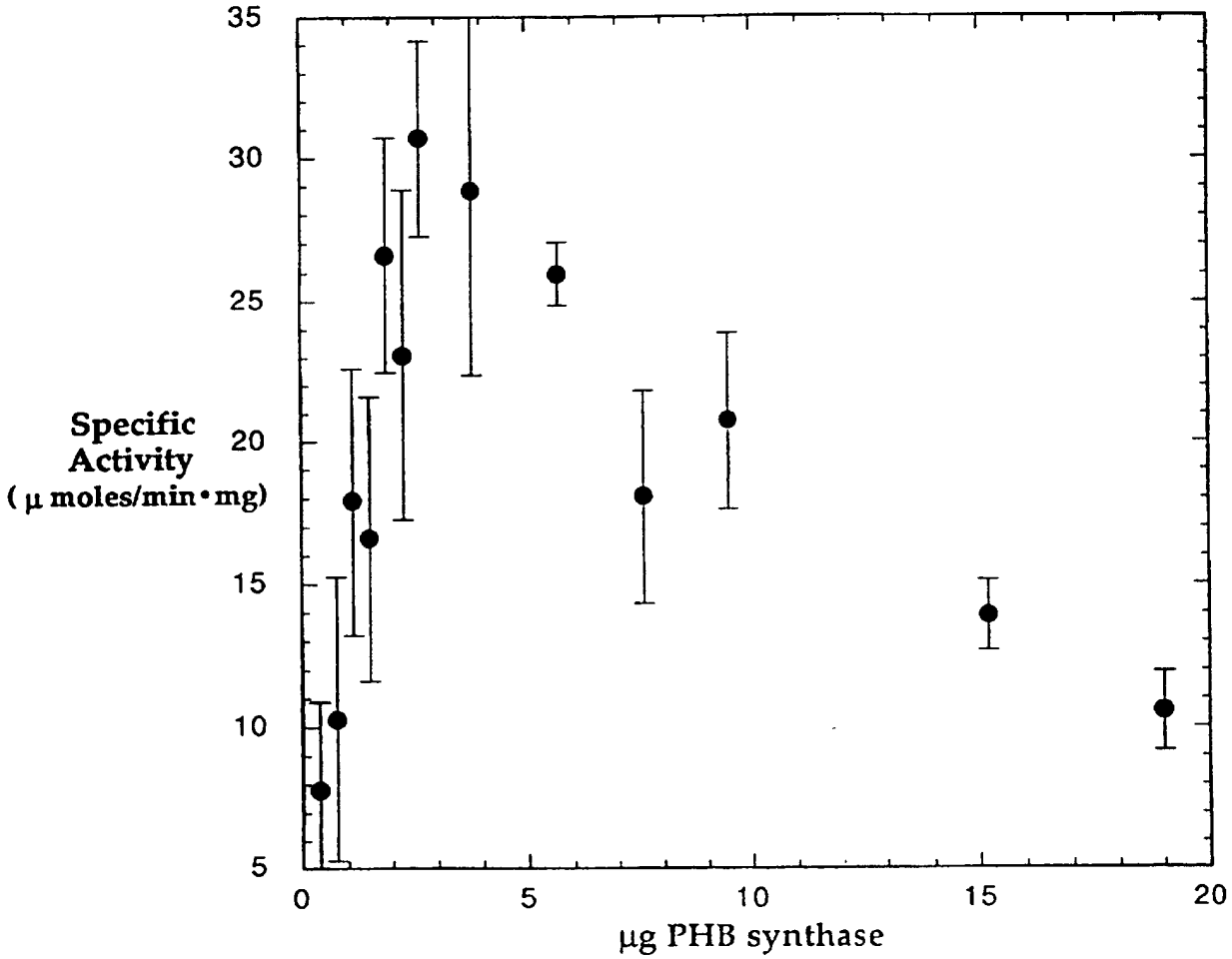

Specific Activity
( μ moles/min • mg)
35
30
25
20
15
10
5
0
5
10
15
20
μg PHB synthase

Figure 14

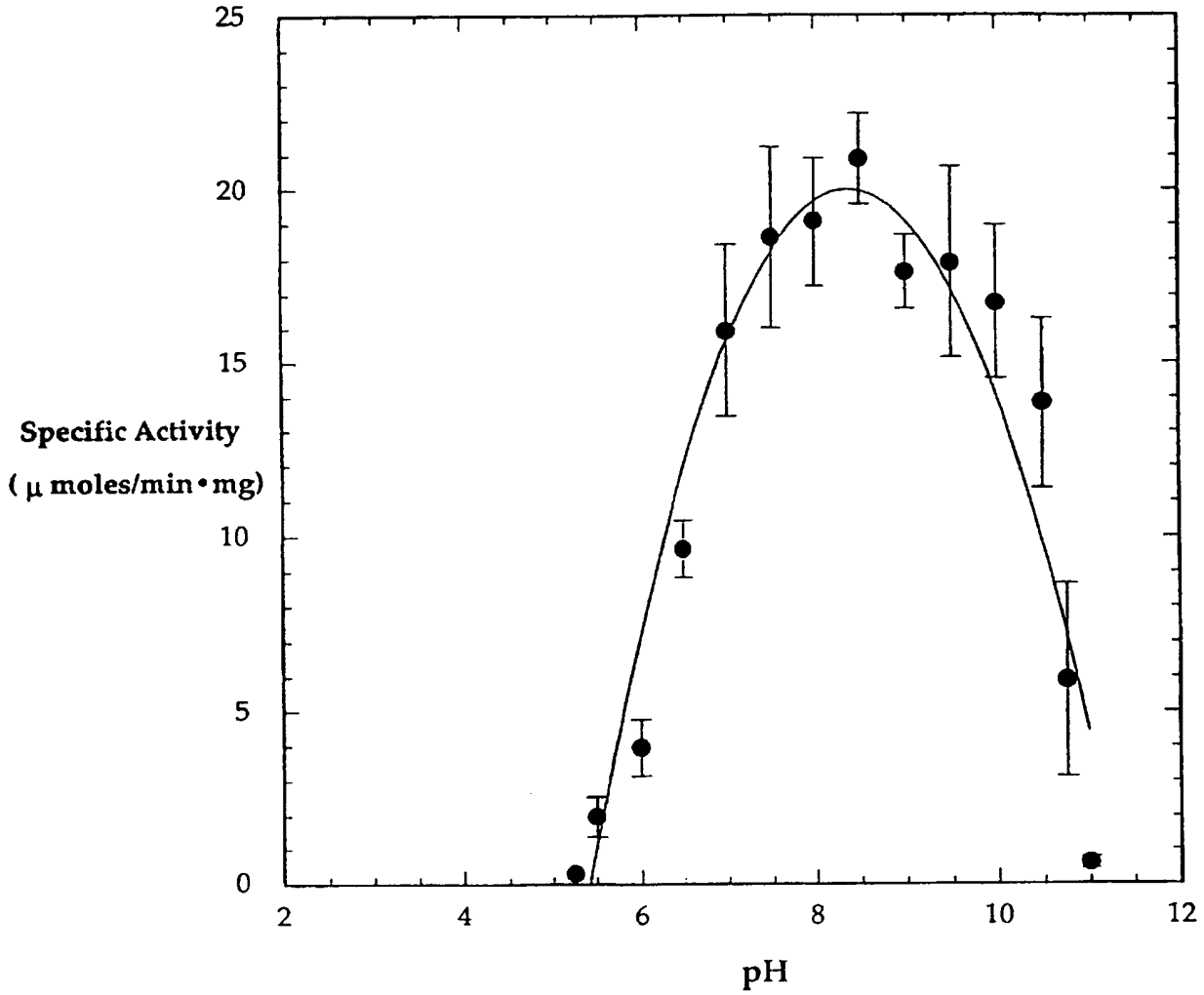
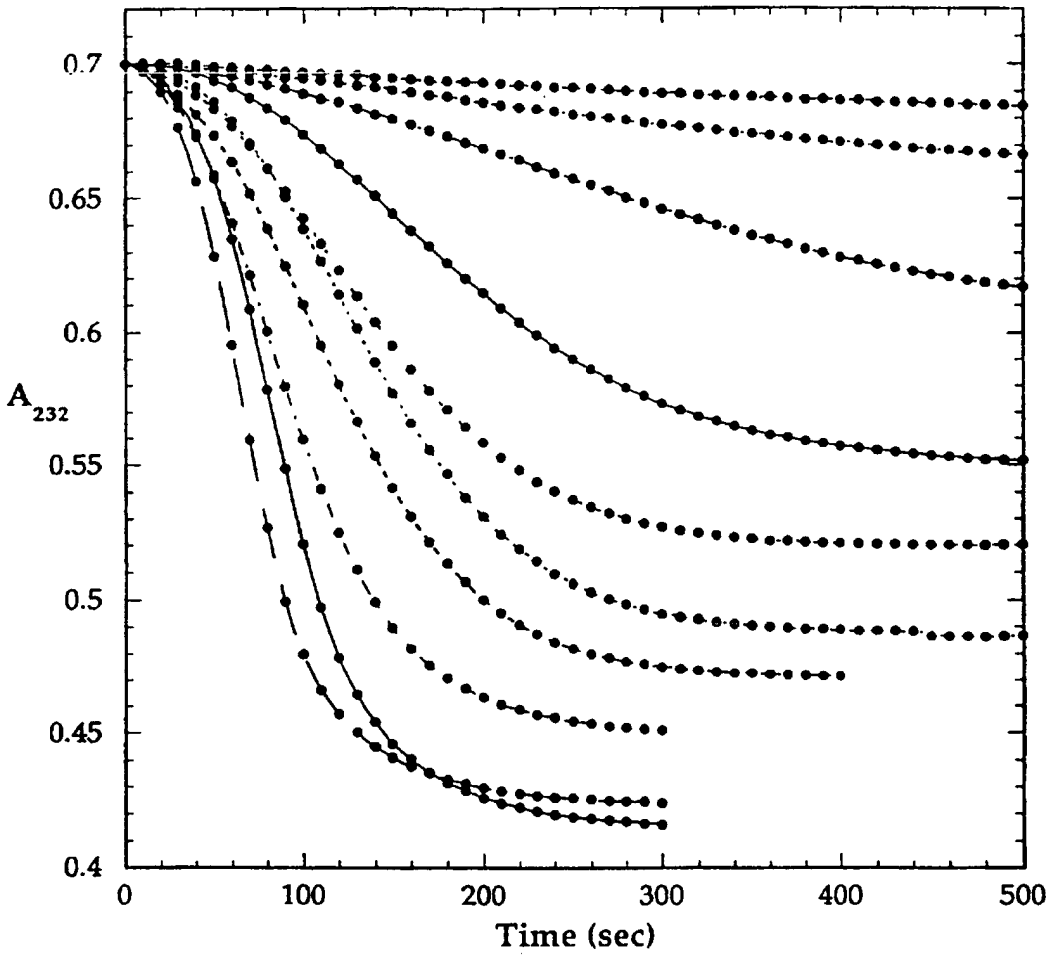


Figure 15



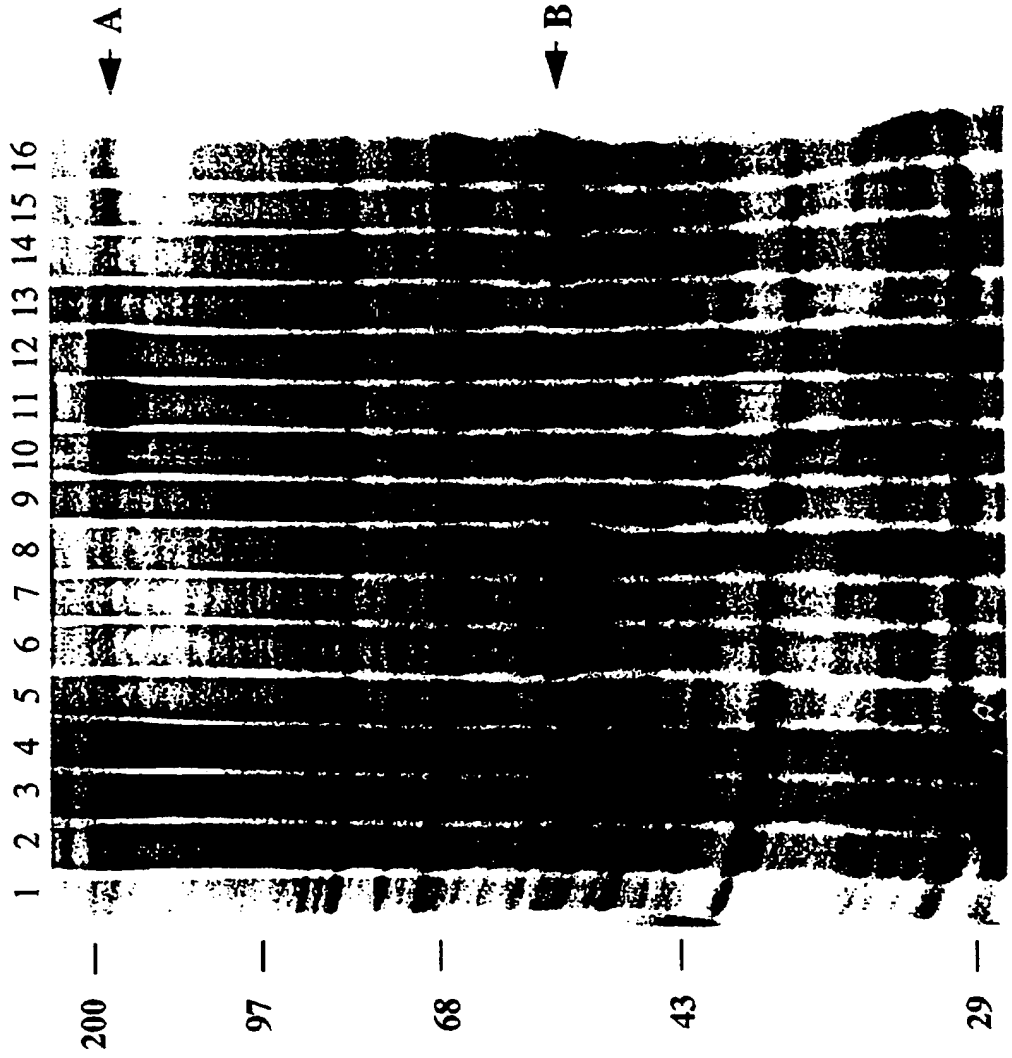



Figure 6

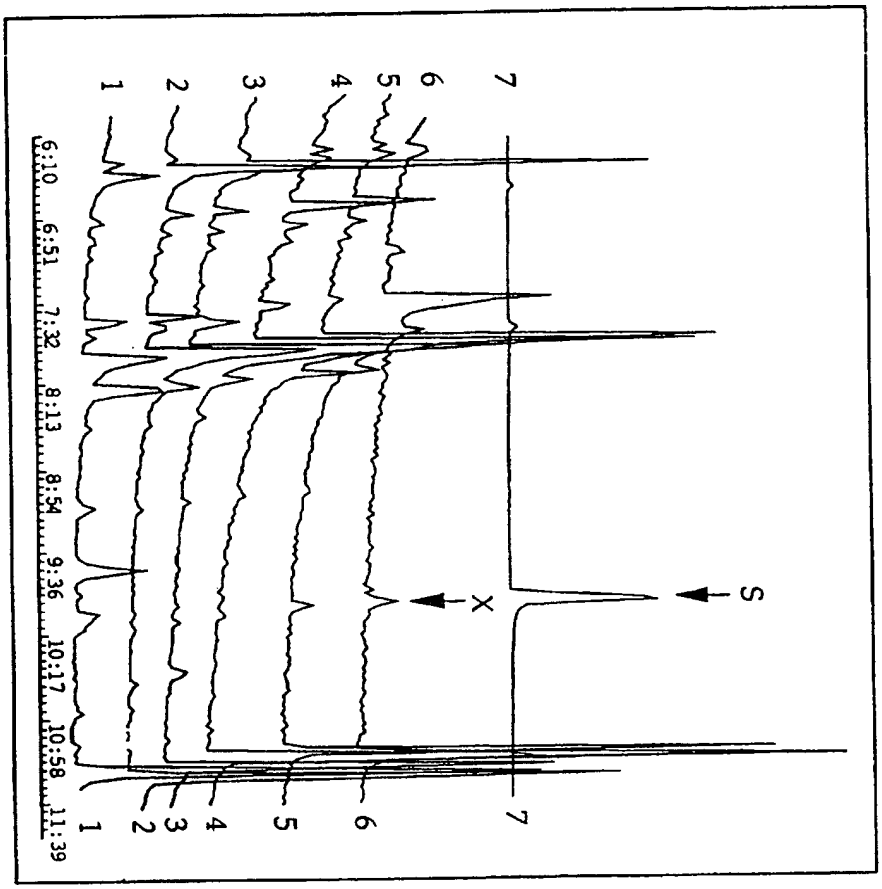



Figure 7

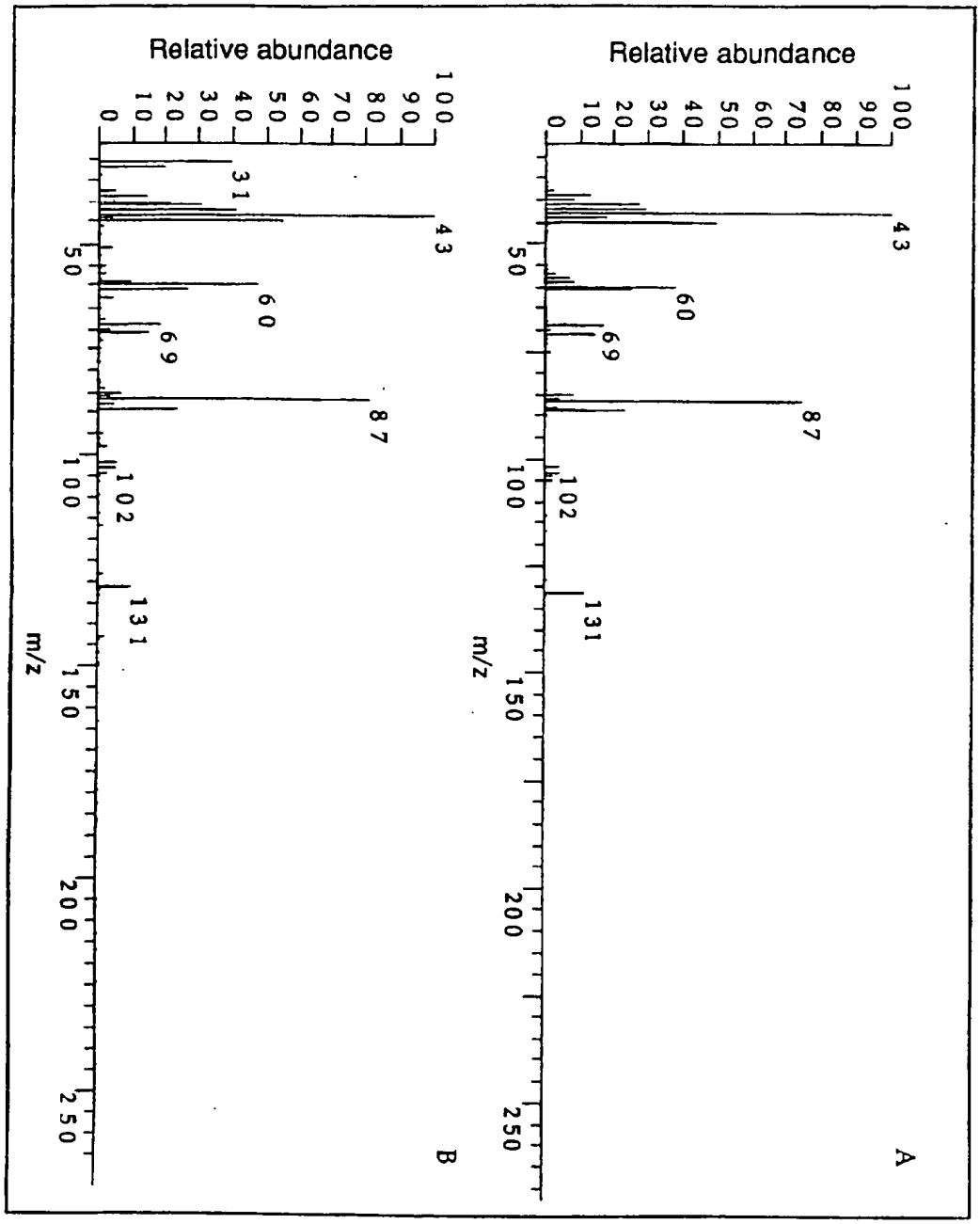

Relative abundance
100
90
80
70
60
50
40
30
20
10
0
43
60
69
87
102
131
50
100
150
200
250
m/z
A
Relative abundance
100
90
80
70
60
50
40
30
20
10
0
31
43
60
69
87
102
131
50
100
150
200
250
m/z
B

Figure 18

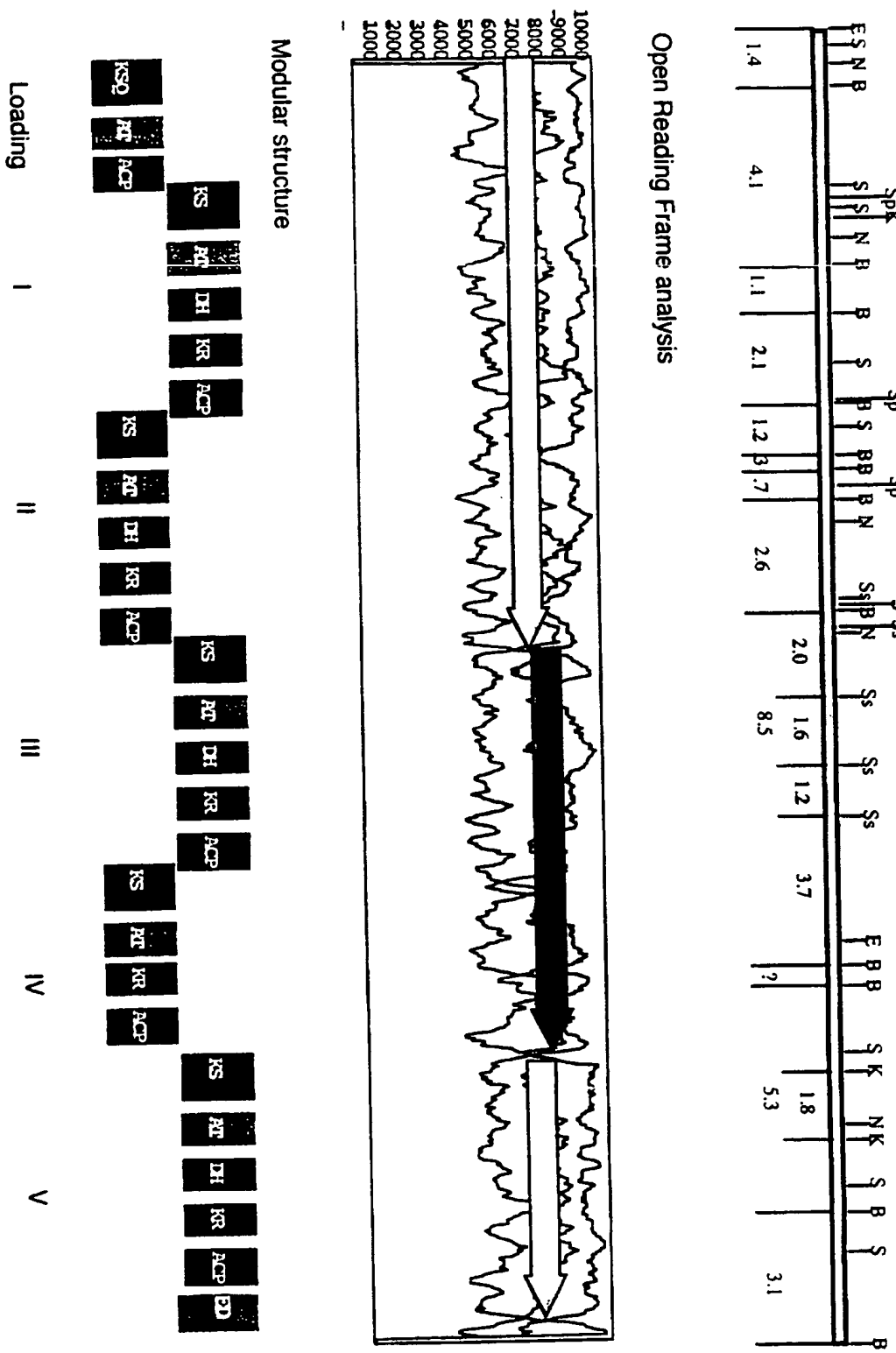

The Map of uep Cluster
Open Reading Frame analysis
Modular structure
Loading
I
II
III
IV
V
KS
AT
DH
KR
ACP
PD
KS2
MT
1.4
4.1
1.1
2.1
1.2
.7
2.6
2.0
1.6
8.5
1.2
3.7
?
1.8
5.3
3.1

Figure 19

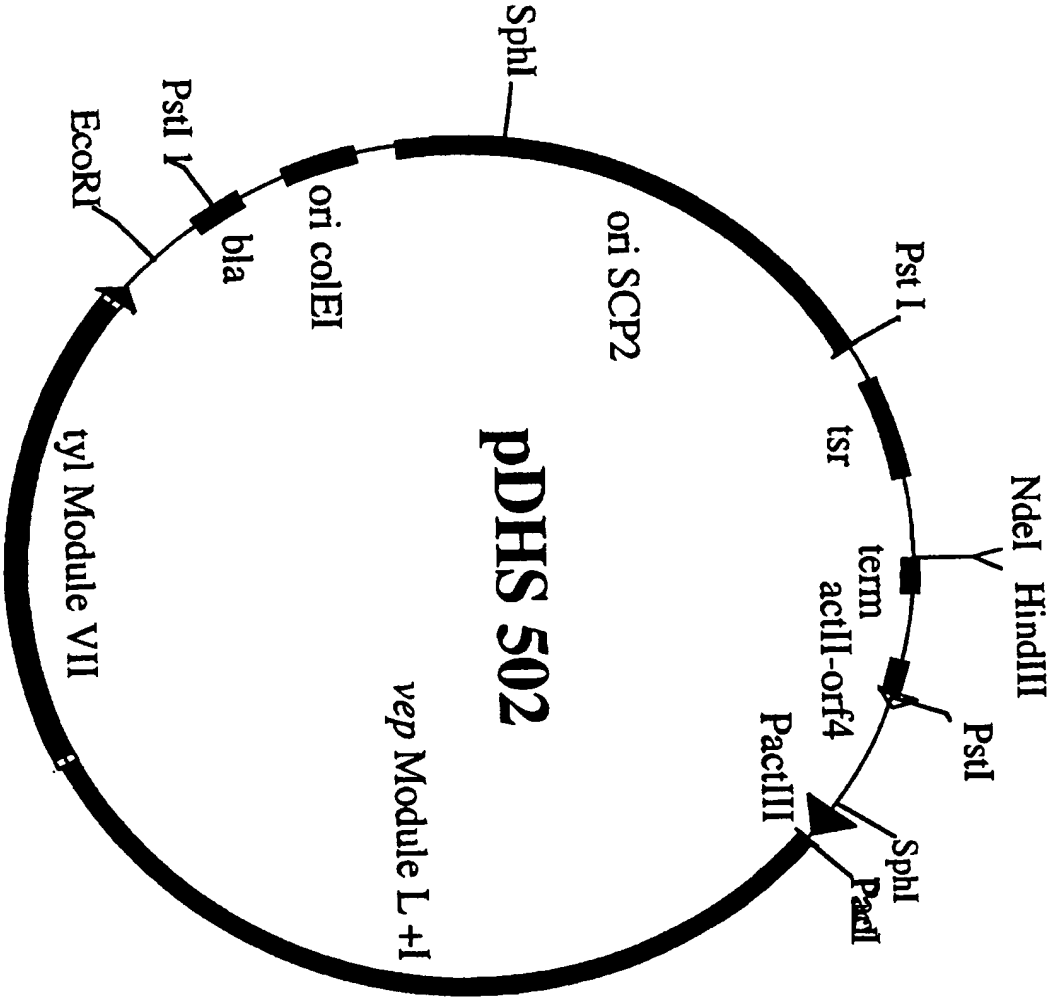

pDHS 502
SphI
ori SCP2
Pst I
tsr
NdeI HindIII
term
actII-orf4
PstI
PactIII
SphI
PacII
vep Module L +I
tyl Module VII
EcoRI
PstI
bla
ori colEI

Figure 20

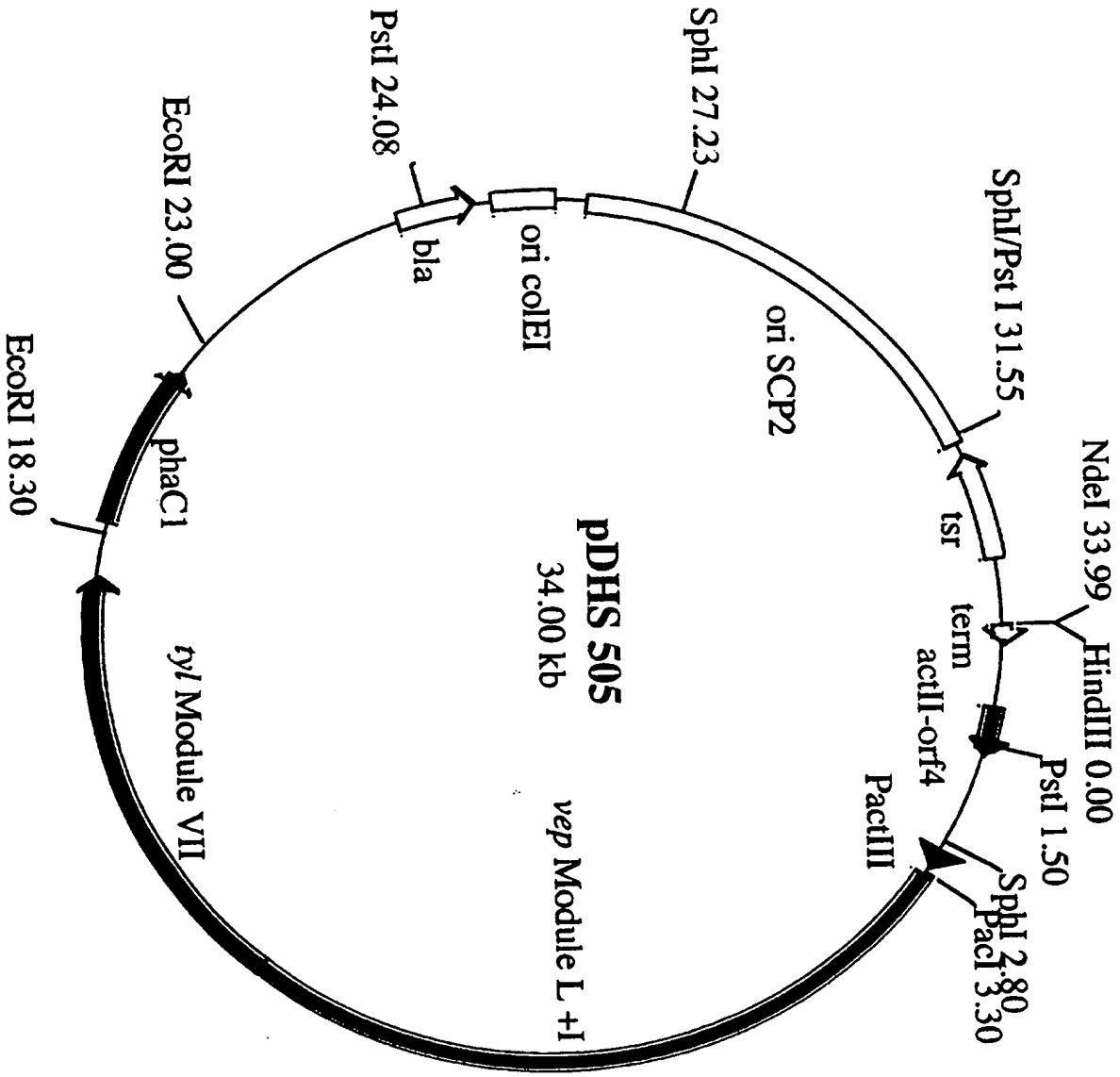
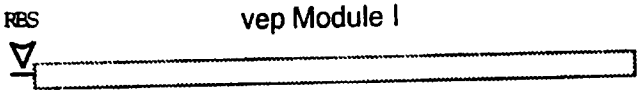

pDHS 505
34.00 kb
NdeI 33.99
HindIII 0.00
PstI 1.50
SphI 2.80
PacI 3.30
SphI/Pst I 31.55
SphI 27.23
PstI 24.08
EcoRI 23.00
EcoRI 18.30
tsr
term
actII-orf4
PactIII
ori SCP2
ori colEI
bla
phaC1
tyl Module VII
vep Module L +I

Figure 21

# pDHS505 Construction Procedure

Restriction map of pDHS505 insert


Major steps in the construction




**1.** A Streptomyces Ribosome Binding Site (RBS) was introduced 6 nucleotides upstreem of the translation start site to enhance gene translation in the host.



**2.** The tyl Module VII was recombined by a BamHI site with the vep Module I to give out a complete polyketide synthase Open Reading Frame (ORF) with a Thioesterase at 3'-end.

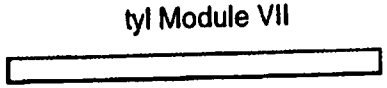
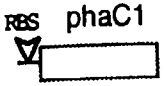
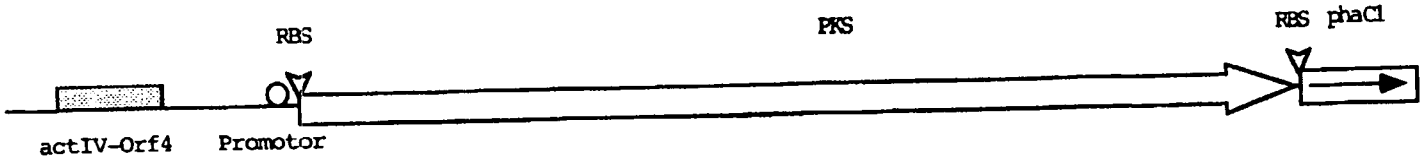



Figure 2c

**3.** The phaC1 gene was transcriptionally coupled with the PKS gene. The second Ribosome Binding Site (RBS) was introduced to facillitate the gene translation.






**4.** The whole expression construct was put under the controle of act promotor and the actII-Orf4 provides an activator which enhances the transcription and expression of the genes.

```
   1 TTAATTAAGGAGGACCATC ATG AAC GAG GCC ATC GCC GTC GTC GGC ATG TCC TGC CGC CTG CCG  64
   1                     M   N   E   A   I   A   V   V   G   M   S   C   R   L   P   15

  65 AAG GCC TCG AAC CCG GCC GCC TTC TGG GAG CTG CTG CGG AAC GGG GAG AGC GCC GTC ACC  124
  16 K   A   S   N   P   A   A   F   W   E   L   L   R   N   G   E   S   A   V   T   35

 125 GAC GTG CCC TCC GGC CGG TGG ACG TCG GTG CTC GGG GGA GCG GAC GCC GAG GAG CCG GCG  184
  36 D   V   P   S   G   R   W   T   S   V   L   G   G   A   D   A   E   E   P   A   55

 185 GAG TCC GGT GTC CGC CGG GGC GGC TTC CTC GAC TCC CTC GAC CTC TTC GAC GCG GCC TTC  244
  56 E   S   G   V   R   R   G   G   F   L   D   S   L   D   L   F   D   A   A   F   75

 245 TTC GGA ATC TCG CCC CGT GAG GCC GCC GCC ATG GAC CCG CAG CAG CGA CTG GTC CTC GAA  304
  76 F   G   I   S   P   R   E   A   A   A   M   D   P   Q   Q   R   L   V   L   E   95

 305 CTC GCC TGG GAG GCG CTG GAG GAC GCC GGA ATC GTC CCC GGC ACC CTC GCC GGA AGC CGC  364
  96 L   A   W   E   A   L   E   D   A   G   I   V   P   G   T   L   A   G   S   R   115

 365 ACC GCC GTC TTC GTC GGC ACC CTG CGG GAC GAC TAC ACG AGC CTC CTC TAC CAG CAC GGC  424
 116 T   A   V   F   V   G   T   L   R   D   D   Y   T   S   L   L   Y   Q   H   G   135

 425 GAG CAG GCC ATC ACC CAG CAC ACC ATG GCG GGC GTG AAC CGG GGC GTC ATC GCC AAC CGC  484
 136 E   Q   A   I   T   Q   H   T   M   A   G   V   N   R   G   V   I   A   N   R   155

 485 GTC TCG TAC CAC CTC GGC CTG CAG GGC CCG AGC CTC ACC GTC GAC GCC GCG CAG TCG TCC  544
 156 V   S   Y   H   L   G   L   Q   G   P   S   L   T   V   D   A   A   Q   S   S   175

 545 TCG CTC GTC GCC GTG CAC CTG GCC TGC GAG TCC CTG CGC GCC GGG GAG TCC ACG ACG GCG  604
 176 S   L   V   A   V   H   L   A   C   E   S   L   R   A   G   E   S   T   T   A   195

 605 CTC GTC GCC GGC GTG AAC CTC AAC ATC CTC GCG GAG AGC GCC GTG ACG GAG GAG CGC TTC  664
 196 L   V   A   G   V   N   L   N   I   L   A   E   S   A   V   T   E   E   R   F   215

 665 GGT GGA CTC TCC CCG GAC GGC ACC GCC TAC ACC TTC GAC GCG CGG GCC AAC GGA TTC GTC  724
 216 G   G   L   S   P   D   G   T   A   Y   T   F   D   A   R   A   N   G   F   V   235

 725 CGG GGC GAG GGC GGC GGA GTC GTC GTA CTC AAG CCG CTC TCC CGC GCC CTC GCC GAC GGC  784
 236 R   G   E   G   G   G   V   V   V   L   K   P   L   S   R   A   L   A   D   G   255

 785 GAC CGT GTC CAC GGC GTC ATC CGC GCC AGC GCC GTC AAC AAC GAC GGA GCC ACC CCG GGT  844
 256 D   R   V   H   G   V   I   R   A   S   A   V   N   N   D   G   A   T   P   G   275

 845 CTC ACC GTG CCC AGC AGG GCC GCC CAG GAG AAG GTG CTG CGC GAG GCG TAC CGG AAG GCG  904
 276 L   T   V   P   S   R   A   A   Q   E   K   V   L   R   E   A   Y   R   K   A   295

 905 GCC CTG GAC CCG TCC GCC GTC CAG TAC GTC GAA CTC CAC GGC ACC GGA ACC CCC GTC GGC  964
 296 A   L   D   P   S   A   V   Q   Y   V   E   L   H   G   T   G   T   P   V   G   315

 965 GAC CCC ATC GAG GCC GCC GCG CTC GGC GCC GTC CTC GGC TCG GCG CGC CCC GCG GAC GAA 1024
 316 D   P   I   E   A   A   A   L   G   A   V   L   G   S   A   R   P   A   D   E   335

1025 CCC CTG CTC GTC GGC TCG GCC AAG ACG AAC GTC GGG CAC CTC GAA GGC GCC GCC GGC ATC 1084
 336 P   L   L   V   G   S   A   K   T   N   V   G   H   L   E   G   A   A   G   I   355

1085 GTC GGC CTC ATC AAG ACG CTC CTC GCG CTC GGC CGG CGC CGG ATC CCG GCG AGC CTC AAC 1144
 356 V   G   L   I   K   T   L   L   A   L   G   R   R   R   I   P   A   S   L   N   375

1145 TTC CGT ACG CCC CAC CCG GAC ATC CCG CTC GAC ACC CTC GGG CTC GAC GTG CCC GAC GGC 1204
 376 F   R   T   P   H   P   D   I   P   L   D   T   L   G   L   D   V   P   D   G   395

1205 CTG CGG GAG TGG CCG CAC CCG GAC CGC GAA CTC CTC GCC GGC GTC AGC TCG TTC GGC ATG 1264
 396 L   R   E   W   P   H   P   D   R   E   L   L   A   G   V   S   S   F   G   M   415

1265 GGC GGC ACC AAC GCC CAC GTC GTC CTC AGC GAA GGC CCC GCC CAG GGC GGC GAG CAG CCC 1324
 416 G   G   T   N   A   H   V   V   L   S   E   G   P   A   Q   G   G   E   Q   P   435

1325 GGC ATC GAT GAG GAG ACC CCC GTC GAC AGC GGG GCC GCA CTG CCC TTC GTC GTC ACC GGC 1384
 436 G   I   D   E   E   T   P   V   D   S   G   A   A   L   P   F   V   V   T   G   455

1385 CGC GGC GGC GAG GCC CTG CGC GCC CAG GCC CGG CGC CTG CAC GAG GCC GTC GAA GCG GAC 1444
 456 R   G   G   E   A   L   R   A   Q   A   R   R   L   H   E   A   V   E   A   D   475
```

Figure 23

```
1445 CCG GAG CTC GCG CCC GCC GCA CTC GCC CGG TCG CTG GTC ACC ACC CGT ACG GTC TTC ACG 1504
 476 P   E   L   A   P   A   A   L   A   R   S   L   V   T   T   R   T   V   F   T   495

1505 CAC CGG TCG GTC GTC CTC GCC CCG GAC CGC GCC CGC CTC CTC GAC GGC CTC GGC GCC CTC 1564
 496 H   R   S   V   V   L   A   P   D   R   A   R   L   L   D   G   L   G   A   L   515

1565 GCC GCC GGG ACG CCC GCG CCC GGC GTG GTC ACC GGC ACC CCC GCC CCC GGG CGC CTC GCC 1624
 516 A   A   G   T   P   A   P   G   V   V   T   G   T   P   A   P   G   R   L   A   535

1625 GTC CTG TTC AGC GGC CAG GGT GCC CAA CGT ACG GGC ATG GGC ATG GAG TTG TAC GCC GCC 1684
 536 V   L   F   S   G   Q   G   A   Q   R   T   G   M   G   M   E   L   Y   A   A   555

1685 CAC CCC GCC TTC GCG ACG GCC TTC GAC GCC GTC GCC GCC GAA CTG GAC CCC CTC CTC GAC 1744
 556 H   P   A   F   A   T   A   F   D   A   V   A   A   E   L   D   P   L   L   D   575

1745 CGG CCC CTC GCC GAA CTC GTC GCG GCG GGC GAC ACC CTC GAC CGC ACC GTC CAC ACA CAG 1804
 576 R   P   L   A   E   L   V   A   A   G   D   T   L   D   R   T   V   H   T   Q   595

1805 CCC GCG CTC TTC GCC GTG GAG GTC GCC CTC CAC CGC CTC GTC GAG TCC TGG GGC GTC ACG 1864
 596 P   A   L   F   A   V   E   V   A   L   H   R   L   V   E   S   W   G   V   T   615

1865 CCC GAC CTG CTC GCC GGC CAC TCC GTC GGC GAG ATC AGC GCC GCC CAC GTC GCC GGG GTC 1924
 616 P   D   L   L   A   G   H   S   V   G   E   I   S   A   A   H   V   A   G   V   635

1925 CTG TCG CTG CGC GAC GCC GCC CGC CTC GTC GCG GCG CGC GGC CGC CTC ATG CAG GCG CTC 1984
 636 L   S   L   R   D   A   A   R   L   V   A   A   R   G   R   L   M   Q   A   L   655

1985 CCC GAG GGC GGC GCG ATG GTC GCG GTC GAG GCG AGC GAG GAG GAA GTG CTT CCG CAC CTC 2044
 656 P   E   G   G   A   M   V   A   V   E   A   S   E   E   E   V   L   P   H   L   675

2045 GCG GGA CGC GAG CGG GAG CTC TCC CTC GCG GCC GTG AAC GGC CCC CGC GCG GTC GTC CTC 2104
 676 A   G   R   E   R   E   L   S   L   A   A   V   N   G   P   R   A   V   V   L   695

2105 GCG GGC GCC GAG CGC GCC GTC CTC GAC GTC GCC GAG CTG CTG CGC GAA CAG GGC CGC CGG 2164
 696 A   G   A   E   R   A   V   L   D   V   A   E   L   L   R   E   Q   G   R   R   715

2165 ACG AAG CGG CTC AGC GTC TCG CAC GCC TTC CAC TCG CCG CTC ATG GAG CCG ATG CTC GAC 2224
 716 T   K   R   L   S   V   S   H   A   F   H   S   P   L   M   E   P   M   L   D   735

2225 GAC TTC CGC CGG GTC GTC GAA GAG CTG GAC TTC CAG GAG CCC CGC GTC GAC GTC GTG TCC 2284
 736 D   F   R   R   V   V   E   E   L   D   F   Q   E   P   R   V   D   V   V   S   755

2285 ACG GTG ACG GGC CTG CCT GTC ACA GCG GGC CAA TGG ACC GAT CCC GAG TAC TGG GTG GAC 2344
 756 T   V   T   G   L   P   V   T   A   G   Q   W   T   D   P   E   Y   W   V   D   775

2345 CAG GTC CGC AGG CCC GTA CGC TTC CTC GAC GCC GTA CGC ACC CTG GAG GAA TCG GGC GCC 2404
 776 Q   V   R   R   P   V   R   F   L   D   A   V   R   T   L   E   E   S   G   A   795

2405 GAC ACC TTC CTG GAG CTC GGT CCC GAC GGG GTC TGC TCC GCG ATG GCG GCG GAC TCC GTA 2464
 796 D   T   F   L   E   L   G   P   D   G   V   C   S   A   M   A   A   D   S   V   815

2465 CGC GAC CAG GAG GCC GCC ACG GCG GTC TCC GCC CTG CGC AAG GGC CGC CCG GAG CCC CAG 2524
 816 R   D   Q   E   A   A   T   A   V   S   A   L   R   K   G   R   P   E   P   Q   835

2525 TCG CTG CTC GCC GCA CTC ACC ACC GTC TTC GTC CGG GGC CAC GAC GTC GAC TGG ACC GCC 2584
 836 S   L   L   A   A   L   T   T   V   F   V   R   G   H   D   V   D   W   T   A   855

2585 GCG CAC GGG AGC ACC GGC ACG GTC AGG GTG CCC CTG CCG ACC TAC GCC TTC CAG CGC GAA 2644
 856 A   H   G   S   T   G   T   V   R   V   P   L   P   T   Y   A   F   Q   R   E   875

2645 CGC CAC TGG TTC GAC GGC GCC GCG CGA ACG GCG GCC CCG CTC ACG GCG GGC CGA TCG GGC 2704
 876 R   H   W   F   D   G   A   A   R   T   A   A   P   L   T   A   G   R   S   G   895

2705 ACC GGT GCG GGC ACC GGC CCG GCC GCG GGT GTG ACG TCG GGC GAG GGC GAG GGC GAG GGC 2764
 896 T   G   A   G   T   G   P   A   A   G   V   T   S   G   E   G   E   G   E   G   915

2765 GAG GGC GCG GGT GCG GGT GGC GGT GAT CGG CCG GCT CGC CAC GAG ACG ACC GAG CGC GTG 2824
 916 E   G   A   G   A   G   G   G   D   R   P   A   R   H   E   T   T   E   R   V   935

2825 CGC GCA CAC GTC GCC GCC GTC CTC GAG TAC GAC GAC CCG ACC CGC GTC GAA CTC GGC CTC 2884
 936 R   A   H   V   A   A   V   L   E   Y   D   D   P   T   R   V   E   L   G   L   955

2885 ACC TTC AAG GAG CTG GGC TTC GAC TCC CTC ATG TCC GTC GAG CTG CGG AAC GCG CTC GTC 2944
 956 T   F   K   E   L   G   F   D   S   L   M   S   V   E   L   R   N   A   L   V   975

2945 GAC GAC ACG GGA CTG CGC CTG CCC AGC GGA CTG CTC TTC GAC CAC CCG ACG CCG CGC GCC 3004
 976 D   D   T   G   L   R   L   P   S   G   L   L   F   D   H   P   T   P   R   A   995
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Figure 23 cont.

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3005 CTC GCC GCC CAC CTG GGC GAC CTG CTC ACC GGC GGC AGC GGC GAG ACC GGA TCG GCC GAC  3064
 996 L   A   A   H   L   G   D   L   L   T   G   G   S   G   E   T   G   S   A   D    1015

3065 GGG ATA CCG CCC GCG ACC CCG GCG GAC ACC ACC GCC GAG CCC ATC GCG ATC ATC GGC ATG  3124
1016 G   I   P   P   A   T   P   A   D   T   T   A   E   P   I   A   I   I   G   M    1035

3125 GCC TGC CGC TAC CCC GGC GGC GTC ACC TCC CCC GAG GAC CTG TGG CGG CTC GTC GCC GAG  3184
1036 A   C   R   Y   P   G   G   V   T   S   P   E   D   L   W   R   L   V   A   E    1055

3185 GGG CGC GAC GCC GTC TCG GGG CTG CCC ACC GAC CGC GGC TGG GAC GAG GAC CTC TTC GAC  3244
1056 G   R   D   A   V   S   G   L   P   T   D   R   G   W   D   E   D   L   F   D    1075

3245 GCC GAC CCC GAC CGC AGC GGC AAG AGC TCG GTC CGC GAG GGC GGA TTC CTG CAC GAC GCC  3304
1076 A   D   P   D   R   S   G   K   S   S   V   R   E   G   G   F   L   H   D   A    1095

3305 GCC CTG TTC GAC GCC GGC TTC TTC GGG ATA TCG CCC CGC GAC CCC CTC GGC ATG GAC CCG  3364
1096 A   L   F   D   A   G   F   F   G   I   S   P   R   E   A   L   G   M   D   P    1115

3365 CAG CAG CGG CTG CTC CTG GAG ACG GCA TGG GAG GCC GTG GAG CGC GCA GGG CTC GAC CCC  3424
1116 Q   Q   R   L   L   L   E   T   A   W   E   A   V   E   R   A   G   L   D   P    1135

3425 GAA GGC CTC AAG GGC AGC CGG ACG GCC GTC TTC GTC GGC GCC ACC GCC CTG GAC TAC GGC  3484
1136 E   G   L   K   G   S   R   T   A   V   F   V   G   A   T   A   L   D   Y   G    1155

3485 CCG CGC ATG CAC GAC GGC GCC GAG GGC GTC GAG GGC CAC CTC CTG ACC GGG ACC ACG CCC  3544
1156 P   R   M   H   D   G   A   E   G   V   E   G   H   L   L   T   G   T   T   P    1175

3545 AGC GTG ATG TCG GGC CGC ATC GCC TAC CAG CTC GGC CTC ACC GGT CCT GCG GTC ACC GTC  3604
1176 S   V   M   S   G   R   I   A   Y   Q   L   G   L   T   G   P   A   V   T   V    1195

3605 GAC ACG GCC TGC TCG TCC TCG CTC GTC GCG CTG CAC CTG GCC GTC CGT TCG CTG CGG CAG  3664
1196 D   T   A   C   S   S   S   L   V   A   L   H   L   A   V   R   S   L   R   Q    1215

3665 GGC GAG TCG AGC CTC GCG CTC GCC GGC GGA GCG ACC GTC ATG TCG ACA CCG GGC ATG TTC  3724
1216 G   E   S   S   L   A   L   A   G   G   A   T   V   M   S   T   P   G   M   F    1235

3725 GTC GAG TTC TCG CGG CAG CGC GGC CTC GCC GCC GAC GGC CGC TCC AAG GCC TTC TCC GAC  3784
1236 V   E   F   S   R   Q   R   G   L   A   A   D   G   R   S   K   A   F   S   D    1255

3785 TCC GCC GAC GGC ACC TCC TGG GCC GAG GGC GTC GGC CTC CTC GTC GTC GAG CGG CTC TCG  3844
1256 S   A   D   G   T   S   W   A   E   G   V   G   L   L   V   V   E   R   L   S    1275

3845 GAC GCC GAG CGC AAC GGC CAC CCC GTG CTC GCC GTG ATC CGG GGC AGC GCG GTC AAC CAG  3904
1276 D   A   E   R   N   G   H   P   V   L   A   V   I   R   G   S   A   V   N   Q    1295

3905 GAC GGC GCC TCC AAC GGG CTC ACC GCC CCC AAC GGC CCG TCC CAG CAG CGC GTC ATC CGA  3964
1296 D   G   A   S   N   G   L   T   A   P   N   G   P   S   Q   Q   R   V   I   R    1315

3965 CAG GCC CTG GCC GAC GCC GGG CTC ACC CCG GCC GAC GTC GAC GCC GTC GAG GCG CAC GGT  4024
1316 Q   A   L   A   D   A   G   L   T   P   A   D   V   D   A   V   E   A   H   G    1335

4025 ACG GGT ACC CGG CTC GGC GAC CCC ATC GAG GCC GAG GCG ATC CTC GGC ACC TAC GGC CGG  4084
1336 T   G   T   R   L   G   D   P   I   E   A   E   A   I   L   G   T   Y   G   R    1355

4085 GAC CGG GGC GAG GGC GCT CCG CTC CAG CTC GGC TCG CTG AAG TCG AAC ATC GGC CAC GCG  4144
1356 D   R   G   E   G   A   P   L   Q   L   G   S   L   K   S   N   I   G   H   A    1375

4145 CAG GCC GCC GCG GGC GTG GGC GGG CTC ATC AAG ATG GTC CTC GCG ATG CGC CAC GGC GTC  4204
1376 Q   A   A   A   G   V   G   G   L   I   K   M   V   L   A   M   R   H   G   V    1395

4205 CTG CCC AGG ACG CTC CAC GTG GAC CGG CCC ACC ACC CGC GTC GAC TGG GAG GCC GGC GGC  4264
1396 L   P   R   T   L   H   V   D   R   P   T   T   R   V   D   W   E   A   G   G    1415

4265 GTC GAG CTC CTC ACC GAG GAG CGG GAG TGG CCG GAG ACG GGC CGC CCG CGC CGC GCG GCG  4324
1416 V   E   L   L   T   E   E   R   E   W   P   E   T   G   R   P   R   R   A   A    1435

4325 ATC TCC TCC TTC GGC ATC AGC GGC ACC AAC GCC CAC ATC GTG GTC GAA CAG GCC CCG GAA  4384
1436 I   S   S   F   G   I   S   G   T   N   A   H   I   V   V   E   Q   A   P   E    1455

4385 GCC GGG GAG GCG GCG GTC ACC ACC ACC GCC CCG GAA GCA GGG GAA GCC GGG GAA GCG GCG  4444
1456 A   G   E   A   A   V   T   T   T   A   P   E   A   G   E   A   G   E   A   A    1475

4445 GAC ACC ACC GCC ACC ACG ACG CCG GCC GCG GTC GGC GTC CCC GAA CCC GTA CGC GCC CCC  4504
1476 D   T   T   A   T   T   T   P   A   A   V   G   V   P   E   P   V   R   A   P    1495

4505 GTC GTG GTC TCC GCG CGG GAC GCC GCC GCC CTG CGC GCC CAG GCC GTT CGG CTG CGG ACC  4564
1496 V   V   V   S   A   R   D   A   A   A   L   R   A   Q   A   V   R   L   R   T    1515
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Figure 23 cont.

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4565 TTC CTC GAC GGC CGA CCG GAC GTC ACC GTC GCC GAC CTC GGA CGC TCG CTG GCC GCC CGT  4624
1516 F   L   D   G   R   P   D   V   T   V   A   D   L   G   R   S   L   A   A   R    1535

4625 ACC GCC TTC GAG CAC AAG GCC GCC CTC ACC ACC GCC ACC AGG GAC GAG CTG CTC GCC GGG  4684
1536 T   A   F   E   H   K   A   A   L   T   T   A   T   R   D   E   L   L   A   G    1555

4685 CTC GAC GCC CTC GGC CGC GGG GAG CAA GCC ACG GGC CTG GTC ACC GGC GAA CCG GCC AGG  4744
1556 L   D   A   L   G   R   G   E   Q   A   T   G   L   V   T   G   E   P   A   R    1575

4745 GCC GGA CGC ACG GCC TTC CTG TTC ACC GGC CAG GGA GCG CAG CGC GTC GCC ATG GGC GAG  4804
1576 A   G   R   T   A   F   L   F   T   G   Q   G   A   Q   R   V   A   M   G   E    1595

4805 GAA CTG CGC GCC GCG CAC CCC GTG TTC GCC GCC GCC CTC GAC ACC GTG TAC GCG GCC CTC  4864
1596 E   L   R   A   A   H   P   V   F   A   A   A   L   D   T   V   Y   A   A   L    1615

4865 GAC CGT CAC CTC GAC CGG CCG CTG CGG GAG ATC GTC GCC GCC GGG GAG GAG CTG GAC CTC  4924
1616 D   R   H   L   D   R   P   L   R   E   I   V   A   A   G   E   E   L   D   L    1635

4925 ACC GCG TAC ACC CAG CCC GCC CTC TTC GCC TTC GAG GTG GCG CTG TTC CGC CTC CTC GAA  4984
1636 T   A   Y   T   Q   P   A   L   F   A   F   E   V   A   L   F   R   L   L   E    1655

4985 CAC CAC GGC CTC GTC CCC GAC CTG CTC ACC GGC CAC TCC GTC GGC GAG ATC GCC GCC GCG  5044
1656 H   H   G   L   V   P   D   L   L   T   G   H   S   V   G   E   I   A   A   A    1675

5045 CAC GTC GCC GGT GTC CTC TCC CTC GAC GAC GCC GCA CGT CTC GTC ACC GCC CGC GGC CGG  5104
1676 H   V   A   G   V   L   S   L   D   D   A   A   R   L   V   T   A   R   G   R    1695

5105 CTC ATG CAG TCG GCC CGC GAG GGC GGC GCG ATG ATC GCC GTG CAG GCG GGC GAG GCC GAG  5164
1696 L   M   Q   S   A   R   E   G   G   A   M   I   A   V   Q   A   G   E   A   E    1715

5165 GTC GTC GAG TCC CTG AAG GGC TAC GAG GGC AGG GTC GCC GTC GCC GCC GTC AAC GGA CCC  5224
1716 V   V   E   S   L   K   G   Y   E   G   R   V   A   V   A   A   V   N   G   P    1735

5225 ACC GCC GTG GTC GTC TCC GGC GAC GCG GAC GCC GCC GAG GAG ATC CGC GCC GTA TGG GCG  5284
1736 T   A   V   V   V   S   G   D   A   D   A   A   E   E   I   R   A   V   W   A    1755

5285 GGA CGC GGC CGG CGC ACC CGC AGG CTG CGC GTC AGC CAC GCC TTC CAC TCC CCG CAC ATG  5344
1756 G   R   G   R   R   T   R   R   L   R   V   S   H   A   F   H   S   P   H   M    1775

5345 GAC GAC GTC CTC GAC GAG TTC CTC CGG GTC GCC GAG GGC CTG ACC TTC GAG GAG CCG CGG  5404
1776 D   D   V   L   D   E   F   L   R   V   A   E   G   L   T   F   E   E   P   R    1795

5405 ATC CCC GTC GTC TCC ACG GTC ACC GGC GCG CTC GTC ACG TCC GGC GAG CTC ACC TCG CCC  5464
1796 I   P   V   V   S   T   V   T   G   A   L   V   T   S   G   E   L   T   S   P    1815

5465 GCG TAC TGG GTC GAC CAG ATC CGG CGG CCC GTG CGC TTC CTG GAC GCC GTC CGC ACC CTG  5524
1816 A   Y   W   V   D   Q   I   R   R   P   V   R   F   L   D   A   V   R   T   L    1835

5525 GCC GCC CAG GAC GCG ACC GTC CTC GTC GAG ATC GGC CCC GAC GCC GTC CTC ACG GCA CTC  5584
1836 A   A   Q   D   A   T   V   L   V   E   I   G   P   D   A   V   L   T   A   L    1855

5585 GCC GAG GAG GCT CTC GCG CCC GGC ACG GAC GCC CCG GAC GCC CGG GAC GTC ACG GTC GTC  5644
1856 A   E   E   A   L   A   P   G   T   D   A   P   D   A   R   D   V   T   V   V    1875

5645 CCG CTG CTG CGC GCG GGG CGC CCC GAG CCC GAG ACC CTC GCC GCC GGT CTC GCG ACC GCC  5704
1876 P   L   L   R   A   G   R   P   E   P   E   T   L   A   A   G   L   A   T   A    1895

5705 CAT GTC CAC GGC GCA CCC TTG GAC CGG GCG TCG TTC TTC CCG GAC GGG CGC CGC ACG GAC  5764
1896 H   V   H   G   A   P   L   D   R   A   S   F   F   P   D   G   R   R   T   D    1915

5765 CTG CCC ACG TAC GCC TTC CGG CGC GAG CAC TAC TGG CTG ACG CCC GAG GCC CGT ACG GAC  5824
1916 L   P   T   Y   A   F   R   R   E   H   Y   W   L   T   P   E   A   R   T   D    1935

5825 GCC CGC GCA CTC GGC TTC GAC CCG GCG CGG CAC CCG CTG CTG ACG ACC ACG GTC GAG GTC  5884
1936 A   R   A   L   G   F   D   P   A   R   H   P   L   L   T   T   T   V   E   V    1955

5885 GCC GGC GGC GAC GGC GTC CTG CTG ACC GGC CGT CTC TCC CTG ACC GAC CAG CCC TGG CTG  5944
1956 A   G   G   D   G   V   L   L   T   G   R   L   S   L   T   D   Q   P   W   L    1975

5945 GCC GAC CAC ATG GTC AAC GGC GCC GTC CTG TTG CCG GCC ACC GCC TTC CTG GAG CTC GCC  6004
1976 A   D   H   M   V   N   G   A   V   L   L   P   A   T   A   F   L   E   L   A    1995

6005 CTC GCG GCG GGC GAC CAC GTC GGG GCG GTC CGG GTG GAG GAA CTC ACC CTC GAA GCG CCG  6064
1996 L   A   A   G   D   H   V   G   A   V   R   V   E   E   L   T   L   E   A   P    2015

6065 CTC GTC CTG CCC GAG CGG GGC GCC GTC CGC ATC CAG GTC GGC GTG AGC GGC GAC GGC GAG  6124
2016 L   V   L   P   E   R   G   A   V   R   I   Q   V   G   V   S   G   D   G   E    2035
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Figure 23 Cont.

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6125 TCG CCG GCC GGG CGC ACC TTC GGT GTG TAC AGC ACC CCC GAC TCC GGC GAC ACC GGT GAC  6184
2036 S   P   A   G   R   T   F   G   V   Y   S   T   P   D   S   G   D   T   G   D    2055

6185 GAC GCG CCC CGG GAG TGG ACC CGC CAT GTC TCC GGC GTA CTC GGC GAA GGG GAC CCG GCC  6244
2056 D   A   P   R   E   W   T   R   H   V   S   G   V   L   G   E   G   D   P   A    2075

6245 ACG GAG TCG GAC CAC CCC GGC ACC GAC GGG GAC GGT TCA GCG GCC TGG CCG CCT GCG GCG  6304
2076 T   E   S   D   H   P   G   T   D   G   D   G   S   A   A   W   P   P   A   A    2095

6305 GCG ACC GCC ACA CCC CTC GAC GGC GTC TAC GAC CGG CTC GCG GAG CTC GGC TAC GGA TAC  6364
2096 A   T   A   T   P   L   D   G   V   Y   D   R   L   A   E   L   G   Y   G   Y    2115

6365 GGT CCG GCC TTC CAG GGC CTG ACG GGG CTG TGG CGC GAC GGC GCC GAC ACG CTC GCC GAG  6424
2116 G   P   A   F   Q   G   L   T   G   L   W   R   D   G   A   D   T   L   A   E    2135

6425 ATC CGG CTG CCC GCG GCG CAG CAC GAG AGC GCG GGG CTC TTC GGC GTA CAC CCG GCG CTG  6484
2136 I   R   L   P   A   A   Q   H   E   S   A   G   L   F   G   V   H   P   A   L    2155

6485 CTC GAC GCG GCG CTC CAC CCG ATC GTC CTG GAG GGC AAC TCA GCT GCC GGT GCC TGT GAC  6544
2156 L   D   A   A   L   H   P   I   V   L   E   G   N   S   A   A   G   A   C   D    2175

6545 GCC GAT ACC GAC GCG ACC GAC CGG ATC CGG CTG CCG TTC GCG TGG GCG GGG GTG ACC CTC  6604
2176 A   D   T   D   A   T   D   R   I   R   L   P   F   A   W   A   G   V   T   L    2195

6605 CAC GCC GAA GGG GCC ACC GCG CTC CGC GTA CGG ATC ACA CCC ACC GGC CCG GAC ACG GTC  6664
2196 H   A   E   G   A   T   A   L   R   V   R   I   T   P   T   G   P   D   T   V    2215

6665 ACG CTC CGC CTC ACC GAC ACC ACC GGT GCG CCC GTG GCC ACC GTG GAG TCC CTG ACC CTG  6724
2216 T   L   R   L   T   D   T   T   G   A   P   V   A   T   V   E   S   L   T   L    2235

6725 CGC GCG GTG GCG AAG GAC CGG CTG GGC ACC ACC GCC GGG CGC GTC GAC GAC GCC CTG TTC  6784
2236 R   A   V   A   K   D   R   L   G   T   T   A   G   R   V   D   D   A   L   F    2255

6785 ACG GTC GTG TGG ACG GAG ACC GGC ACA CCG GAA CCC GCA GGG CGC GGA GCC GTG GAG GTC  6844
2256 T   V   V   W   T   E   T   G   T   P   E   P   A   G   R   G   A   V   E   V    2275

6845 GAG GAA CTC GTC GAC CTC GCC GGC CTC GGC GAC CTC GTG GAG CTC GGC GCC GCG GAC GTC  6904
2276 E   E   L   V   D   L   A   G   L   G   D   L   V   E   L   G   A   A   D   V    2295

6905 GTC CTC CGG GCC GAC CGC TGG ACG CTC GAC GGG GAC CCG TCC GCC GCC GCG CGC ACA GCC  6964
2296 V   L   R   A   D   R   W   T   L   D   G   D   P   S   A   A   A   R   T   A    2315

6965 GTC CGG CGC ACC CTC GCC ATC GTC CAG GAG TTC CTG TCC GAG CCG CGC TTC GAC GGC TCG  7024
2316 V   R   R   T   L   A   I   V   Q   E   F   L   S   E   P   R   F   D   G   S    2335

7025 CGA CTG GTG TGC GTC ACC AGG GGC GCG GTC GCC GCA CTC CCC GGC GAG GAC GTC ACC TCC  7084
2336 R   L   V   C   V   T   R   G   A   V   A   A   L   P   G   E   D   V   T   S    2355

7085 CTC GCC ACC GGC CCC CTC TGG GGC CTC GTC CGC TCC GCC CAG TCC GAG AAC CCG GGA CGC  7144
2356 L   A   T   G   P   L   W   G   L   V   R   S   A   Q   S   E   N   P   G   R    2375

7145 CTG TTC CTC CTG GAC CTG GGT GAA GGC GAA GGC GAG CGC GAC GGA GCC GAG GAG CTG ATC  7204
2376 L   F   L   L   D   L   G   E   G   E   G   E   R   D   G   A   E   E   L   I    2395

7205 CGC GCG GCC ACG GCC GGG GAC GAG CCG CAG CTC GCG GCA CGG GAC GGC CGA CTG CTC GCG  7264
2396 R   A   A   T   A   G   D   E   P   Q   L   A   A   R   D   G   R   L   L   A    2415

7265 CCG AGG CTG GCC CGT ACC GCC GCC CTT TCG AGT GAG GAC ACC GCC GGC GGC GCC GAC CGT  7324
2416 P   R   L   A   R   T   A   A   L   S   S   E   D   T   A   G   G   A   D   R    2435

7325 TTC GGC CCC GAC GGC ACC GTC CTC GTC ACC GGG GGC ACC GGA GGC CTC GGA GCG CTC CTC  7384
2436 F   G   P   D   G   T   V   L   V   T   G   G   T   G   G   L   G   A   L   L    2455

7385 GCC CGC CAC CTC GTG GAG CGT CAC GGG GTG CGC CGG CTG CTG CTG GTG AGC CGC CGC GGG  7444
2456 A   R   H   L   V   E   R   H   G   V   R   R   L   L   L   V   S   R   R   G    2475

7445 GCC GAC GCC CCC GGC GCG GCC GAC CTG GGC GAG GAC CTC GCG GGC CTC GGC GCG GAG GTG  7504
2476 A   D   A   P   G   A   A   D   L   G   E   D   L   A   G   L   G   A   E   V    2495

7505 GCG TTC GCC GCC GCC GAC GCC GCC GAC CGC GAG AGC CTG GCG CGG GCG ATC GCC ACC GTG  7564
2496 A   F   A   A   A   D   A   A   D   R   E   S   L   A   R   A   I   A   T   V    2515

7565 CCC GCC GAG CAT CCG CTG ACG GCC GTC GTG CAC ACG GCG GGA GTC GTC GAC GAC GCG ACG  7624
2516 P   A   E   H   P   L   T   A   V   V   H   T   A   G   V   V   D   D   A   T    2535

7625 GTG GAG GCG CTC ACA CCG GAA CGG CTG GAC GCG GTA CTG CGC CCG AAG GTC GAC GCC GCG  7684
2536 V   E   A   L   T   P   E   R   L   D   A   V   L   R   P   K   V   D   A   A    2555
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Figure 23 cont.

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7685 TGG AAC CTG CAC GAG CTC ACC AAG GAC CTG CGG CTC GAC GCC TTC GTC CTC TTC TCC TCC  7744
2556 W   N   L   H   E   L   T   K   D   L   R   L   D   A   F   V   L   F   S   S   2575

7745 GTC TCC GGC ATC GTC GGC ACC GCC GGC CAG GCC AAC TAC GCG GCG GCC AAC ACG GGC CTC  7804
2576 V   S   G   I   V   G   T   A   G   Q   A   N   Y   A   A   A   N   T   G   L   2595

7805 GAC GCC CTC GCC GCC CAC CGC GCC GCC ACG GGC CTG GCC GCC ACG TCG CTG GCC TGG GGC  7864
2596 D   A   L   A   A   H   R   A   A   T   G   L   A   A   T   S   L   A   W   G   2615

7865 CTC TGG GAC GGC ACG CAC GGC ATG GGC GGC ACG CTC GGC GCC GCC GAC CTC GCC CGC TGG  7924
2616 L   W   D   G   T   H   G   M   G   G   T   L   G   A   A   D   L   A   R   W   2635

7925 AGC CGG GCC GGA ATC ACC CCG CTC ACC CCG CTG CAG GGC CTC GCG CTC TTC GAC GCC GCG  7984
2636 S   R   A   G   I   T   P   L   T   P   L   Q   G   L   A   L   F   D   A   A   2655

7985 GTC GCC AGG GAC GAC GCC CTC CTC GTA CCC GCC GGG CTC CGT CCC ACC GCC CAC CGG GCC  8044
2656 V   A   R   D   D   A   L   L   V   P   A   G   L   R   P   T   A   H   R   G   2675

8045 ACG GAC GGA CAG CCT CCT GCG CTG TGG CGC GGC CTC GTC CGG GCG CGC CCG CGC CGT GCC  8104
2676 T   D   G   Q   P   P   A   L   W   R   G   L   V   R   A   R   P   R   R   A   2695

8105 GCG CGG ACG GCC GCC GAG GCG GCG GAC ACG ACC GGC GGC TGG CTG AGC GGG CTC GCC GCA  8164
2696 A   R   T   A   A   E   A   A   D   T   T   G   G   W   L   S   G   L   A   A   2715

8165 CAG TCC CCC GAG GAG CGG CGC AGC ACA GCC GTC ACG CTC GTG ACG GGT GTC GTC GCG GAC  8224
2716 Q   S   P   E   E   R   R   S   T   A   V   T   L   V   T   G   V   V   A   D   2735

8225 GTC CTC GGG CAC GCC GAC TCC GCC GCG GTC GGG GCG GAG CGG TCC TTC AAG GAC CTC GGC  8284
2736 V   L   G   H   A   D   S   A   A   V   G   A   E   R   S   F   K   D   L   G   2755

8285 TTC GAC TCC CTG GCC GGG GTG GAG CTC CGC AAC CGG CTG AAC GCC GCC ACC GGC CTG CGG  8344
2756 F   D   S   L   A   G   V   E   L   R   N   R   L   N   A   A   T   G   L   R   2775

8345 CTC CCC GCG ACC ACG GTC TTC GAC CAT CCC TCG CCG GCC GCG CTC GCG TCC CAT CTC CTC  8404
2776 L   P   A   T   T   V   F   D   H   P   S   P   A   A   L   A   S   H   L   L   2795

8405 GCC CAG GTG CCC GGG TTG AAG GAG GGG ACG GCG GCG ACC GCG ACC GTC GTG GCC GAG CGG  8464
2796 A   Q   V   P   G   L   K   E   G   T   A   A   T   A   T   V   V   A   E   R   2815

8465 GGC GCT TCC TTC GGT GAC CGT GCG ACC GAC GAC GAT CCG ATC GCG ATC GTG GGC ATG GCA  8524
2816 G   A   S   F   G   D   R   A   T   D   D   D   P   I   A   I   V   G   M   A   2835

8525 TGC CGC TAT CCG GGT GGT GTG TCG TCG CCG GAG GAC CTG TGG CGG CTG GTG GCC GAG GGG  8584
2836 C   R   Y   P   G   G   V   S   S   P   E   D   L   W   R   L   V   A   E   G   2855

8585 ACG GAC GCG ATC AGC GAG TTC CCC GTC AAC CGC GGC TGG GAC CTG GAG AGC CTC TAC GAC  8644
2856 T   D   A   I   S   E   F   P   V   N   R   G   W   D   L   E   S   L   Y   D   2875

8645 CCG GAT CCC GAG TCG AAG GGC ACC ACG TAC TGC CGG GAG GGC GGG TTC CTG GAA GGC GCC  8704
2876 P   D   P   E   S   K   G   T   T   Y   C   R   E   G   G   F   L   E   G   A   2895

8705 GGT GAC TTC GAC GCC GCC TTC TTC GGC ATC TCG CCG CGC GAG GCC CTG GTG ATG GAC CCG  8764
2896 G   D   F   D   A   A   F   F   G   I   S   P   R   E   A   L   V   M   D   P   2915

8765 CAG CAG CGG CTG CTG CTG GAG GTG TCC TGG GAG GCG CTG GAA CGC GCG GGC ATC GAC CCG  8824
2916 Q   Q   R   L   L   L   E   V   S   W   E   A   L   E   R   A   G   I   D   P   2935

8825 TCC TCG CTG CGC GGC AGC CGC GGT GGT GTC TAC GTG GGC GCC GCG CAC GGC TCG TAC GCC  8884
2936 S   S   L   R   G   S   R   G   G   V   Y   V   G   A   A   H   G   S   Y   A   2955

8885 TCC GAT CCC CGG CTG GTG CCC GAG GGC TCG GAG GGC TAT CTG CTG ACC GGC AGC GCC GAC  8944
2956 S   D   P   R   L   V   P   E   G   S   E   G   Y   L   L   T   G   S   A   D   2975

8945 GCG GTG ATG TCC GGC CGC ATC TCC TAC GCG CTC GGT CTC GAA GGA CCG TCC ATG ACG GTG  9004
2976 A   V   M   S   G   R   I   S   Y   A   L   G   L   E   G   P   S   M   T   V   2995

9005 GAG ACG GCC TGC TCC TCC TCG CTG GTG GCG CTG CAT CTG GCG GTA CGG GCG CTG CGG CAC  9064
2996 E   T   A   C   S   S   S   L   V   A   L   H   L   A   V   R   A   L   R   H   3015

9065 GGC GAG TGC GGG CTC GCG CTG GCG GGC GGG GTG GCG GTG ATG GCC GAT CCG GCG GCG TTC  9124
3016 G   E   C   G   L   A   L   A   G   G   V   A   V   M   A   D   P   A   A   F   3035

9125 GTG GAG TTC TCC CGG CAG AAG GGG CTG GCC GCC GAC GGC CGC TGC AAG GCG TTC TCG GCC  9184
3036 V   E   F   S   R   Q   K   G   L   A   A   D   G   R   C   K   A   F   S   A   3055

9185 GCC GCC GAC GGC ACC GGC TGG GCC GAG GGC GTC GGC GTG CTC GTC CTG GAG CGG CTG TCG  9244
3056 A   A   D   G   T   G   W   A   E   G   V   G   V   L   V   L   E   R   L   S   3075
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Figure 23 cont.

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9245 GAC GCG CGC CGC GCG GGG CAC ACG GTC CTC GGC CTG GTC ACC GGC ACC GCG GTC AAC CAG  9304
3076 D   A   R   R   A   G   H   T   V   L   G   L   V   T   G   T   A   V   N   Q    3095

9305 GAC GGT GCC TCC AAC GGG CTG ACC GCG CCC AAC GGC CCA GCC CAG CAA CGC GTC ATC GCC  9364
3096 D   G   A   S   N   G   L   T   A   P   N   G   P   A   Q   Q   R   V   I   A    3115

9365 GAG GCG CTC GCC GAC GCC GGG CTG TCC CCG GAG GAC GTG GAC GCG GTC GAG GCG CAC GGC  9424
3116 E   A   L   A   D   A   G   L   S   P   E   D   V   D   A   V   E   A   H   G    3135

9425 ACC GGC ACC CGG CTC GGC GAC CCC ATC GAG GCC GGG GCG CTG CTC GCC GCC TCC GGA CGG  9484
3136 T   G   T   R   L   G   D   P   I   E   A   G   A   L   L   A   A   S   G   R    3155

9485 AAC CGT TCC GGC GAC CAC CCG CTG TGG CTC GGC TCG CTG AAG TCC AAC ATC GGG CAT GCC  9544
3156 N   R   S   G   D   H   P   L   W   L   G   S   L   K   S   N   I   G   H   A    3175

9545 CAG GCC GCC GCC GGT GTC GGC GGC GTC ATC AAG ATG CTC CAG GCG CTG CGG CAC GGC TTG  9604
3176 Q   A   A   A   G   V   G   G   V   I   K   M   L   Q   A   L   R   H   G   L    3195

9605 CTG CCC CGC ACC CTC CAC GCC GAC GAG CCG ACC CCG CAT GCC GAC TGG AGC TCC GGC CGG  9664
3196 L   P   R   T   L   H   A   D   E   P   T   P   H   A   D   W   S   S   G   R    3215

9665 GTA CGG CTG CTC ACC TCC GAG GTG CCG TGG CAG CGG ACC GGC CGG CCC CGG CGG ACC GGG  9724
3216 V   R   L   L   T   S   E   V   P   W   Q   R   T   G   R   P   R   R   T   G    3235

9725 GTG TCC GCC TTC GGC GTC GGC GGC ACC AAT GCC CAT GTC GTC CTC GAA GAG GCA CCC GCC  9784
3236 V   S   A   F   G   V   G   G   T   N   A   H   V   V   L   E   E   A   P   A    3255

9785 CCG CCC GCG CCG GAA CCG GCC GGG GAG GCC CCC GGC GGC TCC CGC GCC GCA GAA GGG GCG  9844
3256 P   P   A   P   E   P   A   G   E   A   P   G   G   S   R   A   A   E   G   A    3275

9845 GAA GGG CCC CTG GCC TGG GTG GTC TCC GGA CGC GAC GAG CCG GCC CTG CGG TCC CAG GCC  9904
3276 E   G   P   L   A   W   V   V   S   G   R   D   E   P   A   L   R   S   Q   A    3295

9905 CGG CGG CTC CGC GAC CAC CTC TCC CGC ACC CCC GGG GCC CGC CCG CGT GAC ATC GCC TTC  9964
3296 R   R   L   R   D   H   L   S   R   T   P   G   A   R   P   R   D   I   A   F    3315

9965 TCC CTC GCC GCC ACG CGC GCA GCC TTT GAC CAC CGC GCC GTG CTG ATC GGC TCG GAC GGG 10024
3316 S   L   A   A   T   R   A   A   F   D   H   R   A   V   L   I   G   S   D   G    3335

10025 GCC GAA CTC GCC GCC GCC CTG GAC GCG TTG GCC GAA GGA CGC GAC GGT CCG GCG GTG GTG 10084
3336  A   E   L   A   A   A   L   D   A   L   A   E   G   R   D   G   P   A   V   V    3355

10085 CGC GGA GTC CGC GAC CGG GAC GGC AGG ATG GCC TTC CTC TTC ACC GGG CAG GGC AGC CAG 10144
3356  R   G   V   R   D   R   D   G   R   M   A   F   L   F   T   G   Q   G   S   Q    3375

10145 CGC GCC GGG ATG GCC CAC GAC CTG CAT GCC GCC CAT ACC TTC TTC GCG TCC GCC CTC GAC 10204
3376  R   A   G   M   A   H   D   L   H   A   A   H   T   F   F   A   S   A   L   D    3395

10205 GAG GTG ACG GAC CGT CTC GAC CCG CTG CTC GGC CGG CCG CTC GGC GCG CTG CTG GAC GCC 10264
3396  E   V   T   D   R   L   D   P   L   L   G   R   P   L   G   A   L   L   D   A    3415

10265 CGA CCC GGC TCG CCC GAA GCG GCA CTC CTG GAC CGG ACC GAG TAC ACC CAG CCG GCG CTC 10324
3416  R   P   G   S   P   E   A   A   L   L   D   R   T   E   Y   T   Q   P   A   L    3435

10325 TTC GCC GTC GAG GTG GCG CTC CAC CGG CTG CTG GAG CAC TGG GGG ATG CGC CCC GAC CTG 10384
3436  F   A   V   E   V   A   L   H   R   L   L   E   H   W   G   M   R   P   D   L    3455

10385 CTG CTG GGG CAC TCG GTG GGC GAA CTG GCG GCC GCC CAC GTC GCG GGT GTG CTC GAT CTC 10444
3456  L   L   G   H   S   V   G   E   L   A   A   A   H   V   A   G   V   L   D   L    3475

10445 CAC GAC GCC TGC GCG CTG GTG GCC GCC CGC GGC AGG CTG ATG CAG CGC CTG CCG CCC GGC 10504
3476  D   D   A   C   A   L   V   A   A   R   G   R   L   M   Q   R   L   P   P   G    3495

10505 GGC GCG ATG GTC TCC GTG CGG GCC GGC GAG GAC GAG GTC CGC GCA CTG CTG GCC GGC CGC 10564
3496  G   A   M   V   S   V   R   A   G   E   D   E   V   R   A   L   L   A   G   R    3515

10565 GAG GAC GCC GTC TGC GTC GCC GCG GTG AAC GGC CCC CGG TCG GTG GTG ATC TCC GGC GCG 10624
3516  E   D   A   V   C   V   A   A   V   N   G   P   R   S   V   V   I   S   G   A    3535

10625 GAG GAA GCG GTG GCC GAG GCG GCG GCG CAG CTC GCC GGA CGA GGC CGC CGC ACC AGG CGG 10684
3536  E   E   A   V   A   E   A   A   A   Q   L   A   G   R   G   R   R   T   R   R    3555

10685 CTC CGC GTC GCG CAC GCC TTC CAC TCA CCC CTG ATG GAC GGC ATG CTC GCC GGA TTC CGG 10744
3556  L   R   V   A   H   A   F   H   S   P   L   M   D   G   M   L   A   G   F   R    3575

10745 GAG GTC GCC GCC GGC CTG CGC TAC CGG GAA CCG GAG CTG ACG GTC GTC TCC ACG GTC ACG 10804
3576  E   V   A   A   G   L   R   Y   R   E   P   E   L   T   V   V   S   T   V   T    3595
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Figure 23 cont.

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10805 GGG CGG CCC GCC CGC CCC GGT GAA CTC ACC GGC CCC GAC TAC TGG GTG GCC CAG GTC CGT 10864
 3596 G   R   P   A   R   P   G   E   L   T   G   P   D   Y   W   V   A   Q   V   R   3615

10865 GAG CCC GTG CGC TTC GCG GAC GCG GTC CGC ACG GCA CAC CGC CTC GGA GCC CGC ACC TTC 10924
 3616 E   P   V   R   F   A   D   A   V   R   T   A   H   R   L   G   A   R   T   F   3635

10925 CTG GAG ACC GGC CCG GAC GGC GTG CTG TGC GGC ATG GCA GAG GAG TGC CTG GAG GAC GAC 10984
 3636 L   E   T   G   P   D   G   V   L   C   G   M   A   E   E   C   L   E   D   D   3655

10985 ACC GTG GCC CTG CTG CCG GCG ATC CAC AAG CCC GGC ACC GCG CCG CAC GGT CCG GCG GCT 11044
 3656 T   V   A   L   L   P   A   I   H   K   P   G   T   A   P   H   G   P   A   A   3675

11045 CCC GGC GCG CTG CGG GCG GCC GCC GCC GCG TAC GGC CGG GGC GCC CGG GTG GAC TGG GCC 11104
 3676 P   G   A   L   R   A   A   A   A   A   Y   G   R   G   A   R   V   D   W   A   3695

11105 GGG ATG CAC GCC GAC GGC CCC GAG GGG CCG GCC CGC CGC GTC GAA CTG CCC GTC CAC GCC 11164
 3696 G   M   H   A   D   G   P   E   G   P   A   R   R   V   E   L   P   V   H   A   3715

11165 TTC CGG CAC CGC CGC TAC TGG CTC GCC CCG GGC CGC GCG GCG GAC ACC GAC GAC TGG ATG 11224
 3716 F   R   H   R   R   Y   W   L   A   P   G   R   A   A   D   T   D   D   W   M   3735

11225 TAC CGG ATC GGC TGG GAC CGG CTG CCG GCT GTG ACC GGC GGG GCC CGG ACC GCC GGC CGC 11284
 3736 Y   R   I   G   W   D   R   L   P   A   V   T   G   G   A   R   T   A   G   R   3755

11285 TGG CTG GTG ATC CAC CCC GAC AGC CCG CGC TGC CGG GAG CTG TCC GGC CAC GCC GAA CGC 11344
 3756 W   L   V   I   H   P   D   S   P   R   C   R   E   L   S   G   H   A   E   R   3775

11345 GCG CTG CGC GCC GCG GGC GCG AGC CCC GTA CCG CTG CCC GTG GAC GCT CCG GCC GCC GAC 11404
 3776 A   L   R   A   A   G   A   S   P   V   P   L   P   V   D   A   P   A   A   D   3795

11405 CGG GCG TCC TTC GCG GCA CTG CTG CGC TCC GCC ACC GGA CCT GAC ACA CGA GGT GAC ACA 11464
 3796 R   A   S   F   A   A   L   L   R   S   A   T   G   P   D   T   R   G   D   T   3815

11465 GCC GCG CCC GTG GCC GGT GTG CTG TCG CTG CTG TCC GAG GAG GAT CGG CCC CAT CGC CAG 11524
 3816 A   A   P   V   A   G   V   L   S   L   L   S   E   E   D   R   P   H   R   Q   3835

11525 CAC GCC CCG GTA CCC GCC GGG GTC CTG GCG ACG CTG TCC CTG ATG CAG GCT ATG GAG GAG 11584
 3836 H   A   P   V   P   A   G   V   L   A   T   L   S   L   M   Q   A   M   E   E   3855

11585 GAG GCG GTG GAG GCT CGC GTG TGG TGC GTC TCC CGC GCC GCG GTC GCC GCC GCC GAC CGG 11644
 3856 E   A   V   E   A   R   V   W   C   V   S   R   A   A   V   A   A   A   D   R   3875

11645 GAA CGG CCC GTC GGC GCG GGC GCC GCC CTG TGG GGG CTG GGG CGG GTG GCC GCC CTG GAA 11704
 3876 E   R   P   V   G   A   G   A   A   L   W   G   L   G   R   V   A   A   L   E   3895

11705 CGC CCC ACC CGG TGG GGC GGT CTC GTG GAC CTG CCC GCC TCG CCC GGT GCG GCG CAC TGG 11764
 3896 R   P   T   R   W   G   G   L   V   D   L   P   A   S   P   G   A   A   H   W   3915

11765 GCG GCC GCC GTG GAA CGG CTC GCC GGT CCC GAG GAC CAG ATC GCC GTG CGC GCG TCC GGC 11824
 3916 A   A   A   V   E   R   L   A   G   P   E   D   Q   I   A   V   R   A   S   G   3935

11825 AGT TGG GGC CGG CGC CTC ACC AGG CTG CCG CGC GAC GGC GGC GGC CGG ACG GCC GCA CCC 11884
 3936 S   W   G   R   R   L   T   R   L   P   R   D   G   G   G   R   T   A   A   P   3955

11885 GCG TAC CGG CCG CGC GGC ACG GTG CTC GTC ACC GGT GGC ACC GGC GCG CTC GGC GGG CAT 11944
 3956 A   Y   R   P   R   G   T   V   L   V   T   G   G   T   G   A   L   G   G   H   3975

11945 CTC GCC CGC TGG CTC GCC GCG GCG GGC GCC GAA CAC CTG GCG CTC ACC AGC CGC CGG GGC 12004
 3976 L   A   R   W   L   A   A   A   G   A   E   H   L   A   L   T   S   R   R   G   3995

12005 CCG GAC GCG CCC GGC GCC GCC GGA CTC GAG GCC GAA CTC CTC CTC CTG GCC GCC AAG GTG 12064
 3996 P   D   A   P   G   A   A   G   L   E   A   E   L   L   L   L   G   A   K   V   4015

12065 ACG TTC GCC GCC TGC GAC ACC GCC GAC CGC GAC GGC CTC GCC CGG GTC CTG CGG GCG ATA 12124
 4016 T   F   A   A   C   D   T   A   D   R   D   G   L   A   R   V   L   R   A   I   4035

12125 CCG GAG GAC ACC CCG CTC ACC GCG GTG TTC CAC GCC GCG GGC GTA CCG CAG GTC ACG CCG 12184
 4036 P   E   D   T   P   L   T   A   V   F   H   A   A   G   V   P   Q   V   T   P   4055

12185 CTG TCC CGT ACC TCG CCC GAG CAC TTC GCC GAC GTG TAC GCG GGC AAG GCG GCG GGC GCC 12244
 4056 L   S   R   T   S   P   E   H   F   A   D   V   Y   A   G   K   A   A   G   A   4075

12245 GCG CAC CTG GAC GAA CTG ACC CGC GAA CTC GGC GCC GGA CTC GAC GCG TTC GTC CTC TAC 12304
 4076 A   H   L   D   E   L   T   R   E   L   G   A   G   L   D   A   F   V   L   Y   4095

12305 TCC TCC GGC GCC GGC GTC TGG GGC AGC GCC GGC CAG GGT GCC TAC GCC GCC GCC AAC GCC 12364
 4096 S   S   G   A   G   V   W   G   S   A   G   Q   G   A   Y   A   A   A   N   A   4115
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Figure 23 cont.

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12365 GCC CTG GAC GCG CTC GCC CGG CGC CGT GCG GCG GAC GGA CTC CCC GCC ACC TCC ATC GCC 12424
 4116 A   L   D   A   L   A   R   R   R   A   A   D   G   L   P   A   T   S   I   A   4135

12425 TGG GGC GTG TGG GGC GGC GGC GGT ATG GGG GCC GAC GAG GCG GGC GCG GAG TAT CTG GGC 12484
 4136 W   G   V   W   G   G   G   G   M   G   A   D   E   A   G   A   E   Y   L   G   4155

12485 CGG CGC GGT ATG CGC CCC ATG GCA CCG GTC TCC GCG CTC CGG GCG ATG GCC ACC GCC ATC 12544
 4156 R   R   G   M   R   P   M   A   P   V   S   A   L   R   A   M   A   T   A   I   4175

12545 GCC TCC GGG GAA CCC TGC CCC ACC GTC ACC CAC ACC GAC TGG GAG CGC TTC GGC GAG GGC 12604
 4176 A   S   G   E   P   C   P   T   V   T   H   T   D   W   E   R   F   G   E   G   4195

12605 TTC ACC GCC TTC CGG CCC AGC CCT CTG ATC GCG GGG CTC GGC ACG CCG GGC GGC GGC CGG 12664
 4196 F   T   A   F   R   P   S   P   L   I   A   G   L   G   T   P   G   G   G   R   4215

12665 GCG GCG GAG ACC CCC GAG GAG GGG AAC GCC ACC GCT GCG GCG GAC CTC ACC GCC CTG CCG 12724
 4216 A   A   E   T   P   E   E   G   N   A   T   A   A   A   D   L   T   A   L   P   4235

12725 CCC GCC GAA CTC CGC ACC GCG CTG CGC GAG CTG GTG CGA GCC CGG ACC GCC GCG GCG CTC 12784
 4236 P   A   E   L   R   T   A   L   R   E   L   V   R   A   R   T   A   A   A   L   4255

12785 GGC CTC GAC GAC CCG GCC GAG GTC GCC GAG GGC GAA CGG TTC CCC GCC ATG GGC TTC GAC 12844
 4256 G   L   D   D   P   A   E   V   A   E   G   E   R   F   P   A   M   G   F   D   4275

12845 TCC CTG GCC ACC GTA CGG CTG CGC CGC GGA CTC GCC TCG GCC ACG GGC CTC GAC CTG CCC 12904
 4276 S   L   A   T   V   R   L   R   R   G   L   A   S   A   T   G   L   D   L   P   4295

12905 CCC GAT CTG CTC TTC GAC CGG GAC ACC CCG GCC GCG CTC GCC GCC CAC CTG GCC GAA CTG 12964
 4296 P   D   L   L   F   D   R   D   T   P   A   A   L   A   A   H   L   A   E   L   4315

12965 CTC GCC ACC GCA CGG GAC CAC GGA CCC GGC GGC CCC GGG ACC GGT GCC GCG CCG GCC GAT 13024
 4316 L   A   T   A   R   D   H   G   P   G   G   P   G   T   G   A   A   P   A   D   4335

13025 GCC GGA AGC GGC CTG CCG GCC CTC TAC CGG GAG GCC GTC CGC ACC GGC CGG GCC GCG GAA 13084
 4336 A   G   S   G   L   P   A   L   Y   R   E   A   V   R   T   G   R   A   A   E   4355

13085 ATG GCC GAA CTG CTC GCC GCC GCT TCC CGG TTC CGC CCC GCC TTC GGG ACG GCG GAC CGG 13144
 4356 M   A   E   L   L   A   A   A   S   R   F   R   P   A   F   G   T   A   D   R   4375

13145 CAG CCG GTG GCC CTC GTG CCG CTG GCC GAC GGC GCG GAG GAC ACC GGG CTC CCG CTG CTC 13204
 4376 Q   P   V   A   L   V   P   L   A   D   G   A   E   D   T   G   L   P   L   L   4395

13205 GTG GGC TGC GCC GGG ACG GCG GTG GCC TCC GGC CCG GTG GAG TTC ACC GCC TTC GCC GGA 13264
 4396 V   G   C   A   G   T   A   V   A   S   G   P   V   E   F   T   A   F   A   G   4415

13265 GCG CTG GCG GAC CTC CCG GCG GCG GCC CCG ATG GCC GCG CTG CCG CAG CCC GGC TTT CTG 13324
 4416 A   L   A   D   L   P   A   A   A   P   M   A   A   L   P   Q   P   G   F   L   4435

13325 CCG GGA GAA CGA GTC CCG GCC ACC CCG GAG GCA TTG TTC GAG GCC CAG GCG GAA GCG CTG 13384
 4436 P   G   E   R   V   P   A   T   P   E   A   L   F   E   A   Q   A   E   A   L   4455

13385 CTG CGC TAC GCG GCC GGC CGG CCC TTC GTG CTG CTG GGG CAC TCC GCC GGC GCC AAC ATG 13444
 4456 L   R   Y   A   A   G   R   P   F   V   L   L   G   H   S   A   G   A   N   M   4475

13445 GCC CAC GCC CTG ACC CGT CAT CTG GAG GCG AAC GGT GGC GGC CCC GCA GGG CTG GTG CTC 13504
 4476 A   H   A   L   T   R   H   L   E   A   N   G   G   G   P   A   G   L   V   L   4495

13505 ATG GAC ATC TAC ACC CCC GCC GAC CCC GGC GCG ATG GGC GTC TGG CGG AAC GAC ATG TTC 13564
 4496 M   D   I   Y   T   P   A   D   P   G   A   M   G   V   W   R   N   D   M   F   4515

13565 CAG TGG GTC TGG CGG CGC TCG GAC ATC CCC CCG GAC GAC CAC CGC CTC ACG GCC ATG GGC 13624
 4516 Q   W   V   W   R   R   S   D   I   P   P   D   D   H   R   L   T   A   M   G   4535

13625 GCC TAC CAC CGG CTG CTT CTC GAC TGG TCG CCC ACC CCC GTC CGC GCC CCC GTA CTG CAT 13684
 4536 A   Y   H   R   L   L   L   D   W   S   P   T   P   V   R   A   P   V   L   H   4555

13685 CTG CGC GCC GCG GAA CCC ATG GGC GAC TGG CCA CCC GGG GAC ACC GGC TGG CAG TCC CAC 13744
 4556 L   R   A   A   E   P   M   G   D   W   P   P   G   D   T   G   W   Q   S   H   4575

13745 TGG GAC GGC GCG CAC ACC ACC GCC GGC ATC CCC GGA AAC CAC TTC ACG ATG ATG ACC GAA 13804
 4576 W   D   G   A   H   T   T   A   G   I   P   G   N   H   F   T   M   M   T   E   4595

13805 CAC GCC TCC GCC GCC GCC CGG CTC GTG CAC GGC TGG CTC GCG GAA CGG ACC CCG TCC GGG 13864
 4596 H   A   S   A   A   A   R   L   V   H   G   W   L   A   E   R   T   P   S   G   4615

13865 CAG GGC GGG TCA CCG TCC CGC GCG GCG GGG AGA GAG GAG AGG CCG TGA ACACGGCAGCCGGCCC 13928
 4616 Q   G   G   S   P   S   R   A   A   G   R   E   E   R   P   *                   4631
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Figure 23 cont.

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13929 GACCGGCACCGCCGCCGGCGGCACCACCGCCCCGGCGGCGGCACACGACCTGTCCCGCGCCGGACGCAGGCTCCAACTCA 14008

14009 CCCGGGCCGCACAGTGGTTCGCCGGCAACCAGGGAGACCCCTACGGG ATG ATC CTG CGC GCC GGC ACC GCC  14079
    1                                                 M   I   L   R   A   G   T   A      8

14080 GAC CCG GCA CCG TAC GAG GAA GAG ATC CCC GGG TAC CGA GCT CGA ATT CTT AAT TAA GGAG 14140
    9 D   P   A   P   Y   E   E   E   I   P   G   Y   R   A   R   I   L   N   *       27

14141 GTCGTAG ATG AGT AAC AAG AAC AAC GAT GAG CTG CAG CGG CAG GCC TCG GAA AAC ACC CTG  14201
    1          M   S   N   K   N   N   D   E   L   Q   R   Q   A   S   E   N   T   L      18

14202 GGG CTG AAC CCG GTC ATC GGT ATC CGC CGC AAA GAC CTG TTG AGC TCG GCA CGC ACC GTG  14261
   19 G   L   N   P   V   I   G   I   R   R   K   D   L   L   S   S   A   R   T   V      38

14262 CTG CGC CAG GCC GTC CGC CAA CCG CTG CAC AGC GCC AAG CAT GTG GCC CAC TTT GGC CTG  14321
   39 L   R   Q   A   V   R   Q   P   L   H   S   A   K   H   V   A   H   F   G   L      58

14322 GAG CTG AAG AAC GTG CTG CTG GGC AAG TCC AGC CTT GCC CCG GAA AGC GAC GAC CGT CGC  14381
   59 E   L   K   N   V   L   L   G   K   S   S   L   A   P   E   S   D   D   R   R      78

14382 TTC AAT GAC CCG GCA TGG AGC AAC AAC CCA CTT TAC CGC CGC TAC CTG CAA ACC TAT CTG  14441
   79 F   N   D   P   A   W   S   N   N   P   L   Y   R   R   Y   L   Q   T   Y   L      98

14442 GCC TGG CGC AAG GAG CTG CAG GAC TGG ATC GGC AAC AGC GAC CTG TCG CCC CAG GAC ATC  14501
   99 A   W   R   K   E   L   Q   D   W   I   G   N   S   D   L   S   P   Q   D   I     118

14502 AGC CGC GGC CAG TTC GTC ATC AAC CTG ATG ACC GAA GCC ATG GCT CCG ACC AAC ACC CTG  14561
  119 S   R   G   Q   F   V   I   N   L   M   T   E   A   M   A   P   T   N   T   L     138

14562 TCC AAC CCG GCA GCA GTC AAA CGC TTC TTC GAA ACC GGC GGC AAG AGC CTG CTC GAT GGC  14621
  139 S   N   P   A   A   V   K   R   F   F   E   T   G   G   K   S   L   L   D   G     158

14622 CTG TCC AAC CTG GCC AAG GAC CTG GTC AAC AAC GGT GGC ATG CCC AGC CAG GTG AAC ATG  14681
  159 L   S   N   L   A   K   D   L   V   N   N   G   G   M   P   S   Q   V   N   M     178

14682 GAC GCC TTC GAG GTG GGC AAG AAC CTG GGC ACC AGT GAA GGC GCC GTG GTG TAC CGC AAC  14741
  179 D   A   F   E   V   G   K   N   L   G   T   S   E   G   A   V   V   Y   R   N     198

14742 GAT GTG CTG GAG CTG ATC CAG TAC AAG CCC ATC ACC GAG CAG GTG CAT GCC CGC CCG CTG  14801
  199 D   V   L   E   L   I   Q   Y   K   P   I   T   E   Q   V   H   A   R   P   L     218

14802 CTG GTG GTG CCG CCG CAG ATC AAC AAG TTC TAC GTA TTC GAC CTG AGC CCG GAA AAG AGC  14861
  219 L   V   V   P   P   Q   I   N   K   F   Y   V   F   D   L   S   P   E   K   S     238

14862 CTG GCA CGC TAC TGC CTG CGC TCG CAG CAG CAG ACC TTC ATC ATC AGC TGG CGC AAC CCG  14921
  239 L   A   R   Y   C   L   R   S   Q   Q   Q   T   F   I   I   S   W   R   N   P     258

14922 ACC AAA GCC CAG CGC GAA TGG GGC CTG TCC ACC TAC ATC GAC GCG CTC AAG GAG GCG GTC  14981
  259 T   K   A   Q   R   E   W   G   L   S   T   Y   I   D   A   L   K   E   A   V     278

14982 GAC GCG GTG CTG GCG ATT ACC GGC AGC AAG GAC CTG AAC ATG CTC GGT GCC TGC TCC GGC  15041
  279 D   A   V   L   A   I   T   G   S   K   D   L   N   M   L   G   A   C   S   G     298

15042 GGC ATC ACC TGC ACG GCA TTG GTC GGC CAC TAT GCC GCC CTC GGC GAA AAC AAG GTC AAT  15101
  299 G   I   T   C   T   A   L   V   G   H   Y   A   A   L   G   E   N   K   V   N     318

15102 GCC CTG ACC CTG CTG GTC AGC GTG CTG GAC ACC ACC ATG GAC AAC CAG GTC GCC CTG TTC  15161
  319 A   L   T   L   L   V   S   V   L   D   T   T   M   D   N   Q   V   A   L   F     338

15162 GTC GAC GAG CAG ACT TTG GAG GCC GCC AAG CGC CAC TCC TAC CAG GCC GGT GTG CTC GAA  15221
  339 V   D   E   Q   T   L   E   A   A   K   R   H   S   Y   Q   A   G   V   L   E     358

15222 GGC AGC GAG ATG GCC AAG GTG TTC GCC TGG ATG CGC CCC AAC GAC CTG ATC TGG AAC TAC  15281
  359 G   S   E   M   A   K   V   F   A   W   M   R   P   N   D   L   I   W   N   Y     378

15282 TGG GTC AAC AAC TAC CTG CTC GGC AAC GAG CCG CCG GTG TTC GAC ATC CTG TTC TGG AAC  15341
  379 W   V   N   N   Y   L   L   G   N   E   P   P   V   F   D   I   L   F   W   N     398

15342 AAC GAC ACC ACG CGC CTG CCG GCC GCC TTC CAC GGC GAC CTG ATC GAA ATG TTC AAG AGC  15401
  399 N   D   T   T   R   L   P   A   A   F   H   G   D   L   I   E   M   F   K   S     418

15402 AAC CCG CTG ACC CGC CCG GAC GCC CTG GAG GTT TGC GGC ACT CCG ATC GAC CTG AAA CAG  15461
  419 N   P   L   T   R   P   D   A   L   E   V   C   G   T   P   I   D   L   K   Q     438

15462 GTC AAA TGC GAC ATC TAC AGC CTT GCC GGC ACC AAC GAC CAC ATC ACC CCG TGG CAG TCA  15521
  439 V   K   C   D   I   Y   S   L   A   G   T   N   D   H   I   T   P   W   Q   S     458
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Figure 23 cont.

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15522 TGC TAC CGC TCG GCG CAC CTG TTC GGC GGC AAG ATC GAG TTC GTG CTG TCC AAC AGC GGC 15581
  459 C   Y   R   S   A   H   L   F   G   G   K   I   E   F   V   L   S   N   S   G   478

15582 CAC ATC CAG AGC ATC CTC AAC CCG CCA GGC AAC CCC AAG GCG CGC TTC ATG ACC GGT GCC 15641
  479 H   I   Q   S   I   L   N   P   P   G   N   P   K   A   R   F   M   T   G   A   498

15642 GAT CGC CCG GGT GAC CCG GTG GCC TGG CAG GAA AAC GCC ACC AAG CAT GCC GAC TCC TGG 15701
  499 D   R   P   G   D   P   V   A   W   Q   E   N   A   T   K   H   A   D   S   W   518

15702 TGG CTG CAC TGG CAA AGC TGG CTG GGC GAG CGT GCC GGC GAG CTG GAA AAG GCG CCG ACC 15761
  519 W   L   H   W   Q   S   W   L   G   E   R   A   G   E   L   E   K   A   P   T   538

15762 CGC CTG GGC AAC CGT GCC TAT GCC GCT GGC GAG GCA TCC CCG GGC ACC TAC GTT CAC GAG. 15821
  539 R   L   G   N   R   A   Y   A   A   G   E   A   S   P   G   T   Y   V   H   E   558

15822 CGT TGA GCTGCAGCGCCGTGGCCACCTGCGGGACGCCACGGTGTTGAATTC                          15872
  559 R   *                                                                        560
```

Figure 23 cont.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20119

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12P 7/62; C12N 9/88, 15/63; C07H 21/04
US CL :435/135, 232, 320.1; 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/135, 232, 320.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y | US 5,371,002 A (DENNIS ET AL.) 06 December 1994, column 9, line 65 through column 10, line 62 and Figure 9. | 4, 5, 22, 25-27, 33-35, 52, 57, 59<br>---<br>1-3, 6-21, 28, 29, 32, 38, 51 |
| Y | FRASER, M.J. The Baculovirus-Infected Insect Cell as a Eukaryotic Gene Expression System. Curr. Top. Microbiol. Immunol. August 1992, Vol. 158, pages 131-172, especially pages 145-158. | 1-3, 6-21, 38 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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03 APRIL 1997

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20119

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | JOSHI, A.N. et al. Construction, Expression, and Characterization of a Mutated Animal Fatty Acid Synthase Deficient in the Dehydrase Function. J. Biol. Chem. 25 October 1993, Vol. 268, No. 30, pages 22508-22513, see whole document. | 13-21, 28, 29-32, 40-49, 51 |
| X | US 5,229,279 A (PEOPLES ET AL) 20 July 1993, column 15, lines 43-68. | 37, 58 |
| Y | DONADIO, S. et al. Organization of the Enzymatic Domains in the Multifunctional Polyketide Synthase Involved in Erythromycin Formation in *Saccharopolyspora erythraea*. Gene. 01 February 1992, Vol. 111, No. 1, pages 51-60, see whole document. | 30, 31, 40-49 |
| Y | BEVITT, D.J. et al. 6-Deoxyerythronolide-B Synthase 2 from *Saccharopolyspora erythraea*. Eur. J. Biochem. February 1992, Vol. 204, No. 1, pages 39-49, see whole document. | 30, 31, 40-49 |
| X | Database Medline on Dialog, US National Library of Medicine, (Bethesda, MD, USA), No. 95187308, HAN, L. et al. 'Cloning and Characterization of Polyketide Synthase Genes for Jadomycin B Biosynthesis in Streptomyces venezuelae ISP5230', abstract, Microbiol., Vol. 140, No. 12, pages 3379-89, December 1994, see entire abstract. | 53 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20119

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS; STN-indices Bioscience Patents
search terms: PHA, PHB, polyhydroxyalkanoate, polyhydroxybutyrate, polyketide, fatty acid synthase, dehydrase, Alcaligenes eutrophus, Saccharopolyspora erythraea, S. venezuelae